ON THE NATURE OF BACTERIA

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BRIEF REVIEW OF CURRENT CONCEPTIONS OF THE LOWER ORGANISMS

The question in regard to the position of bacteria in the organic system has received but little attention in medical bacteriology. This is explainable partly because the solution of the question may seem to be of minor practical significance, partly because its discussion demands so extensive a special study that the physician must not be blamed if he hesitates to pass so far beyond the limits of his field. The reason that I, nevertheless, dare to take up this question is that apparently important observations for its solution have been made by medical bacteriologists, observations that have not received due consideration either in botanical or medical circles. Furthermore, in distinction to many others, I am firmly of the opinion that a better knowledge of the nature of bacteria in the long run cannot but be of importance even in practical medicine.

As this article is intended for physicians it seems well to begin with a presentation of the views of a botanist in regard to the development of the lower organisms and then later take up the different theories about the position of the bacteria. I shall follow Lotsy [1] especially. He points out that there is no sharp line dividing the lower animals and plants, because there are organisms that under certain conditions possess chlorophyl and prepare their own food, and that under other conditions are colorless and take up prepared food in fluid or solid state. In the same individual nutrition may be distinctly "animal" and "vegetable." The discovery of such organisms puts an end for good to the search for a definite difference between the animal and the vegetable kingdom. In looking for lower and lower organisms the flagellates are reached, which possess both animal and vegetable characteristics. The primordial organisms may have been like flagellates; they surely lived in water and were able to prepare their own food. Whether the food was assimilated by photosynthesis or not, that is to say, whether these organisms possessed coloring matter or not, is difficult to decide because there are

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[1] Vorträge über botanische Stammesgeschichte, 1907.

plants without coloring matter that are able to produce organic substances from inorganic.

As the nearest relatives to these hypothetic primordial organisms, Lotsy mentions the so-called Protomastigma, which are colorless organisms without cell wall that swim about by means of cilia. Descendants of these organisms have acquired the power of photosynthesis and of forming coloring matter. In this way arose green, yellowish-brown, red and bluish-green flagellates; from these in turn developed the lower algae and from these the rest of the vegetable kingdom.

How do these flagellates look? Lotsy describes a low form, called Pyramidomonas, and also a somewhat further developed form, Chlamydomonas. Pyramidomonas consists of a mass of protoplasm without any cellulose membrane provided with a nucleus and a so-called chromatophor which is a green organ for photosynthesis. Then there are four cilia, situated on a specially formed head-end. Multiplication takes place by simple longitudinal division while the organism is in motion.

Chlamydomonas is more developed in that it has a cellulose membrane and before division changes into a resting form, from which later arise daughter cells with two or four cilia; these cells reach the same size as the mother cell. Multiplication also takes place in a sexual way, the mother cells give rise to 8-32 small daughter cells, which are not capable of independent development, but which unite with similar cells from another individual and together form new organisms—zygotes developed from the union of two gametes.

The next steps in development go in three directions: (1) the cells, which are formed by sexual reproduction, do not separate but form a motile colony; (2) the daughter cells form a colony, which is stationary; (3) the chlamydomonas cell becomes immotile and grows out to form a many nucleated tube, which later by branching may assume most variable forms. In the higher forms the cell is divided by transverse membranes. These three groups are included under the name "Isokontae" and constitute a part of the green algae. The forms belonging to group 3 are called "Siphonales" by Lotsy. In these the sexual reproduction reaches its highest development as seen, for example, in Vaucheria. This form is made up of a long tubular structure and multiplies sexually; in the latter case there is formed ciliated swarming spores, which rapidly reach maturity. In sexual reproduction the same individual forms two branches, one the male organ (antheridium), the other the female (oogonium). From the antheridium arise spermatozoa, one of

which enters the oogonium and unites therein with the nucleus. The zygote thus formed later surrounds itself with a membrane. In addition to this reproduction through special organs Vaucheria may multiply also in another manner: A loosened fragment from any part of the thallus may grow into a new organism, indeed a protoplasmic mass cut off from the wall of the thread may do the same.

From Isokontae arise the higher plants.

Where the border between Isokontae and Protozoa runs, that is, where the animal kingdom begins, is a matter of difference of opinion. Lotsy places Chlamydomonas in Isokontae, while Döflein [2] places not only Chlamydomonas but also a number of higher Isokontae among the protozoa. This does not mean that Döflein regards them as animals; it simply indicates that zoologists in their efforts to trace the development of these organisms come down to the same group as the botanists.

The course from Isokontae to the higher plants does not interest us at present, and the next step will be to trace the lower fungi from the algae just mentioned.

Lotsy includes the lower sponges under Siphonomycetes and divides them into three groups, Monoblepharideae, Zygomycetes and Oomycetes.

From what forms of Siphonales Siphonomycetes are to be derived cannot be determined accurately. With respect to sexual reproduction, Monoblepherides stand closest to Vaucheria. These fungi are aqueous, and form antheridia with spermatozoa and oogonia with eggs. In both the other groups the sexual mode of reproduction has been changed so that the gametes do not escape and seek the oogonia but the gamentangia copulate. This is dependent on the fact that the fungi have become land plants, hence the spermatazoa no longer have any medium in which to move, and this is true also of the zoosporangia and the swarming spores. Such zoosporangia and motile spores have been found in Monoblepherida, but in the others sporangia with immotile spores have developed. A further development of the sporangium is the conidium, which is formed as the sporangium produces only one spore at the same time as the wall and the membrane of the spore no longer are differentiated.

Siphonomycetes correspond fairly well to the old name Phycomycetes as they were called on account of their similarity to the algae.

As example of fungi belonging to this group may be mentioned Mucoraceae, which have a branching unicellular thallus, which forms

[2] Lehrbuch der Protozoenkunde, 1909.

partly many-spored sporangia, partly gametangia which copulate. Furthermore, Mucoraceae may reproduce themselves by the formation of oidia, gemmae and chlamydospores. The first arise by way of transverse partitions in the originally unicellular mycelium, which thus is divided into many cells. When the thread falls to pieces each cell may multiply by budding like yeast cells, and form mycelium. If the oidia differ from the rest of the mycelium in having either a thicker membrane or a darker color they are called gemmae. Chlamydospores arise in the protoplasm of the mycelium, which becomes clumped, and the clump surrounds itself with a solid membrane.

The older mycologists with Brefeld at the head derived the higher fungi from Phycomycetes.

The higher fungi, which in distinction to the lower are multicellular, are included usually under the name Eumycetes. The two most important groups are Ascomycetes, characterized by an ascus, the principal organ of reproduction consisting of a hypha changed to a sporangium-like structure containing a fixed number of spores, usually eight; and Basidiomycetes, in which the spores consist of conidia fastened to a special conidiopherous structure, the so-called basidium, by means of fine shafts (sterigma). According to Brefeld's system, the ascus is the homologue of the sporangium of Phycomycetes and the basidium with their conidiophores. The evolution is regarded as having come through the so-called Hemiasci and Hemibasidii. One can understand how Lindau could say that the fungi is that group of plants which in course of evolution gradually has lost sexual characteristics. Others are of a different opinion, e.g., Lotsy. They do not believe that the asci are developed from the sporangia. On the basis of cytologic observations they believe that the ascus is comparable with the sporangium in the bracken, which is something entirely different. Lotsy calls the structure diplosporangium. In order to understand the condition better, it may be said that in bracken the gametes are differentiated into eggs and spermatozoa. The bracken develops from the fertilized egg or zygote. Its cells contain 2 x chromozomes if one assumes x chromozomes in one gamete. Lotsy consequently calls bracken the 2 x generation. Sporangia now form that contain mother cells, which through reduction give rise to four spores, which subsequently develop into a prothallium, the x generation, which in turn gives rise to gametes. The mother cells of the spores are called "gonotokontes."

Several investigators have found similar conditions among Ascomycetes. Thus Harper found that in "Sphaerothea" the vegetative mycelial

threads by bulging give rise to oogonia and antheridia, both with one nucleus, the nuclei uniting to form a zygote which in turn gives rise to an ascus. The zygote constitutes 2 x generation and the ascospores are "gones," which form the x generation. The ascus is consequently not a phycomycete-sporangium, because this belongs to the x generation, nor is it a gameteangium, but it is what Lotsy calls a diplosporangium because it belongs to the 2 x generation. In principle the conditions are similar in other Ascomycetes and Basidiomycetes except that in the majority the uniting nuclei are not separated into male and female, but are of the same kind (apogamy). These views, advocated even as early as by de Bary,[3] do not correspond with Brefeld's system. A part of Ascomycetes are regarded as coming from Phycomycetes, among which are found some with alternation in generation similar to that just described, but the so-called hemiasci play no part herein. Hemiasci are, according to Lotsy, of the nature of a simple sporangium. Other Ascomycetes, Uredineae and Basidiomycetes, are regarded as arising from Floridineae or the red algae, but the course of this development is not of interest at this time.

There are two other groups, which it seems necessary to understand, namely, Ustilagineae and Saccharomycetes. The latter are regarded as closely related to Ascomycetes. Some form spores with, and some without, copulation. Lotsy has suggested that Saccharomycetes are reduced Ascomycetes; those which form spores without copulation would correspond to the x generation and the formation of spores would be assumed to take place parthenogenetically. By copulation there would form as in Ascomycetes a 2 x generation, a diplosporangium. In the noncopulating it might be possible that a single spore might be formed of each yeast cell. In reality Saccharomyces without copulation forms four spores, Schizosaccharomyces octosporus and Pombe without copulation form, respectively, 8 and 4 spores. Anything definite in regard to the origin of Saccharomycetes is not known.

As example of Ustilago may be mentioned, U. carbo which is a parasite and causes smut on oats. This fungus lives in the ground as chlamydospores which form a promycelium with few cells out of which bud forth yeast-like conidia. Later these conidia may form a mycelium like the yeast, but they may also in case they enter an oat plant develop into a tubular branching mycelium growing in the fruit. Chlamydospores are formed in the mycelium by a change of the cell walls into mucus while the spore acquires a new membrane. In Brefeld's system

[3] Morphologie der Pilze, 1884.

Ustilagineae and Hemibasidii occupy a place between Phycomycetes and Basidiomycetes. Since the newer investigations just mentioned have deprived them of this place at the same time as the nature of the basidium has been made clear, it has not been possible to classify them satisfactorily. Ustilagineae appear to have some interest in connection with the question under discussion and I shall return to them later. It remains to discuss briefly two other groups of fungi, namely, Fungi imperfecti and Myxomycetes.

The so-called Fungi imperfecti form a large group which could not be assigned any definite place, and consequently wás included in an artificial group. The mycelium of these fungi may be both hyphal and budding. The same kind may appear in both these forms under different conditions.. Fructification, both sporangial and sexual, is absent completely. Multiplication takes place only through spores, which in many kinds arise anywhere in the mycelium and are not gathered together in clearly differentiated conidiophores. Any difference between the spores and vegetative cells that have budded out from the mycelium often cannot be distinguished. A form of spore-formation takes place also as hyphae separated into oidia. The formation of resting cells with thick walls occurs also.

Since of old it has been known that many Fungi imperfecti are simply conidial forms of higher fungi (Basidiomycetes, Ascomycetes, Ustilagineae, and even Phycomycetes), it has been pointed out that Ustilago and Mucor during long periods appeared in the form of budding mycelium, and Mucor with hyphae that could form Chlamydospores or separate into oidia. The same is true in the case of many Ascomycetes and Basidiomycetes. Through exact investigations it has been found that many Fungi imperfecti may be made to form organs of fructification, particularly asci.

In other cases, in which such organs have not been found, this may depend on faulty observation, but it has also been thought that certain Fungi imperfecti do not possess such higher organs, which have been lost in the course of evolution. This conception that at least a part of Fungi imperfecti are reduced higher fungi is expressed by Zopf.

As to Myxomycetes, it may be pointed out that Lotsy classes them with animals rather than with plants. At a certain stage they consist of an ameba-like organism which creeps about by ameboid movement and takes up solid food. This ameba may be able to form spores covered with cellulose from which subsequently new amebas may develop by the

protoplasm creeping out through the membrane and forming a new individual.

A few words in regard to Cyanophyceae may be in order at this point. This is a group of algae which also has been looked on as related to the bacteria. Cyanophyceae, or the bluish-green algae, are called also Schizophyceae because they multiply by fission. They possess a bluish-green coloring matter, as indicated by the name, and nourish themselves by photosynthesis. The cell has no cilia; a nucleus has not been demonstrated definitely; in other respects, however, the cell is highly organized and contains a so-called central body, which is regarded by some as a nucleus, by others as a vacuole containing food in reserve.

VIEWS AS TO THE PLACE OF BACTERIA IN THE SYSTEM

First, it is necessary to understand clearly what is meant by bacteria. It may well happen that organisms, far removed from one another, are designated with this name, and that the bacterial world consequently may be of polyphylogenetic origin. As it would take too much space to enter into the details of this question at this time, I shall content myself by accepting the definition of Meyer,[4] and regard as bacteria the organisms which he calls Eubacteria. Meyer includes here also the group that Lehmann and Neumann designate Actinomycetes.

That bacteria are plants and most closely related to Cyanophyceae was claimed as early as in 1853 by Cohn, who regarded them as the first and simplest division in the organic world and accepted Naegli's designation Schizomycetes, consolidating these with Schizophyceae without, however, regarding bacteria as real fungi. He divided the schizophytes without regard as to whether they contained any coloring matter or not, and in that way created a rather peculiar system. Cohn has had many followers. In opposition to Cohn, Meyer claims that there are many similarities, but many more differences between Cyanophyceae and bacteria, and emphasizes especially that the former develop chlamydospores and the latter endospores, the former lacking cilia. He also points out the absence of a central body. On account of these conditions Meyer does not believe that the bacteria are Cyanophyceae that have been reduced through saprophytism and parasitism. I have reached the same conclusion as Meyer but on other grounds. In regard to the spore question, I cannot assign it such great importance for reasons to which I shall return, and in regard to motility, Meyer himself has

[4] Die Zelle der Bakterien, 1912.

acknowledged, in the discussion of his theory that bacteria belong to Ascomycetes, that cilia have little systematic significance. On the other hand, there is no multiplication by budding in Cyanophyceae nor formation of branching threads which can separate into oidia. This, however, is the case with bacteria and, as we shall see, in large degree. To discuss further the eventual relationship of Cyanophyceae to bacteria does not seem necessary because any such theory would appear false at the moment that it became clear that bacteria are more closely related to Fungi, as I shall show.

While Cohn placed the bacteria lowest in the vegetable kingdom, there are others who regard them as closely related to Flagellatae but lower than these, that is to say, they constitute the organisms that come nearest to the hypothetic primordial organisms. Thus Bütschi [5] points out that the bacteria present great similarity to the lowest flagellates. He places special stress on the power of bacteria to move. This characteristic does not in the bacteria as in the algae concern reproductive cells, but the vegetative phase. As in the flagellates, the cells may divide themselves while in motion. He explains the simpler organization as a reduction on the basis of saprophytism. He regards the endospores of bacteria as the homologs of the "Dauercysten" of the flagellates. This opinion is shared in general by de Bary, and Fischer [6] emphasizes that bacteria constitute the lowest group of protozoa, being the common source of Flagellatae and Cyanophyceae.

On the other hand, A. Meyer seems to be right in thinking that bacteria have little in common with flagellates. He repeats the definition that Klebs gave the flagellates, part of which may be emphasized especially, namely, that they are provided with a definitely differentiated anterior end and a number of pulsating vacuoles and at the same time they divide themselves longitudinally. Meyer mentions a flagellate (Spumella vulgaris) which he thinks most closely resembles bacteria and then shows that the similarity after all is quite minimal. It may be mentioned that this flagellate takes up solid food and possesses pulsating vacuoles.

There remains the question of the relation of bacteria to fungi. It may be pointed out at once that the bacteria naturally are fungi if one means simply thallophytes without chromatophores, but this does not tell us anything concerning their relation to the fungi in reality. Cohn observed a fact that suggested a relation between the bacteria and the

[5] Weitere Ausführungen über den Bau der Cyanophyceen u. Bakterien, 1896.
[6] Vorlesungen über Bakterien, 1897.

fungi, when he discovered that Micrococcus as it multiplies may form a rosary-like structure, which led him to doubt whether it in reality concerned a cellular division and not a kind of budding as seen in Saccharomycetes. In the meantime, he did not consider the matter further but regarded the similarity as external only. Brefeld, on the other hand, believes that the division, which the larger forms of bacteria undergo, is much like the formation of oidia and he regards spore-formation by bacteria and the formation of chlamydospores by Basidiomycetes and Ascomycetes as homologous processes, reaching the conclusion that in the future bacteria may be found to be developmental forms of higher fungi. He points out that there is lacking branching threads, which grow at the points. Migula finds an analogy between the spore formation of bacteria and Saccharomyces. Others are of similar opinion. Meyer himself believes that the bacteria stand close to Ascomycetes and Hemiascomycetes, by the side of which he places the bacteria in his system. He points out that the vegetative body in Ascomycetes and bacteria is a thread, which can divide itself irregularly and show branching with growth at the point. The cell membrane and protoplasm are similar, and the vacuoles are also similar and occupy the same place in the cell which in both cases may contain glycogen, fat, and 'volutin.' In both there may be formation of oidia and a relatively highly developed sporangium. It appears that Meyer regards bacterial spore-formation and the formation of asci and hemiasci as homologous, the asci being of the nature of sporangia.

As early as 1893 Johan-Olsen [7] observed that streptococci and sarcina divided themselves in a manner that could not be regarded as different from the budding of yeasts, and he holds that Almquist, Gasparini, Metschnikoff and Nocard demonstrated that mycelial fungi may be smaller than small bacteria and in certain stages appear as bacteria. He claimed to have seen motile bacteria with endogenous spore-formation give rise to a branching mycelium and he concludes that morphologically bacteria cannot be separated from the fungi.

Almquist [8] made important investigations with respect to the questions now discussed of the organisms of cholera, typhoid fever, and diphtheria, and he found that under certain conditions they would form round bodies by way of budding, bodies which later by continued budding would give rise to similar forms or grow out into rods and threads. He found further that these organisms might assume the form of plas-

[7] Om Sop paa levende Jordbund, 1893.
[8] Zeitschr. f. Hyg. u. Infektionskr., 1917, 83, p. 1.

modia such as had been described earlier by de Bary, Hueppe and others. Almquist regards this as indicating that there are points of connection on the part of these organisms with Saccharomycetes, Myxomycetes and thread fungi. More recently Almqvist and Koraen report that they have observed budding in micrococci, thus confirming Cohn and Johan-Olsen.

Gamaleia, in 1900, expressed the same views as Almquist and emphasized especially that the higher fungi under certain conditions may form oidia that multiply by division, and he regards it as likely that the bacteria are simply oidial forms of fungi belonging to the family Streptothricheae. He has observed also bacterial plasmodia and he regards the mucoid layer, which is found more or less well marked around all bacteria, as the protoplasm, the bacteria themselves being the nuclei. The plasmodium arises from the coalescence of the protoplasm of several bacteria. The formation may be called also zooglea, and in certain cases it is capable of independent motion.

The plasmodium of Gamaleia and Almqvist is placed in the foreground by Löhnis and Smith,[9] according to whom each bacterium passes through a developmental cycle which may be divided into two alternating phases. In one phase the bacterium appears as an organized, in the other as an amorphous, structure. They state that "the amorphous stage has been called the 'symplastic' stage because at this time the living matter previously inclosed in the separate cells undergoes a thorough mixing either by a 'melting together' of the content of many cells which leave the empty cell walls behind them. In the first case a readily stainable, in the latter case an unstainable 'symplasm' is produced." Here it may be pointed out that Hueppe regarded zooglea as a resting stage. Löhnis and Smith describe a number of different types that bacteria may assume when they have organized form, among others a coalescence of two individuals, "conjunction," and gonidia-formation within the cells. Their scheme is very complicated as shown by fig. 1. This scheme was developed on the basis of studies of B. azotobacter. It was found that forty different kinds of bacteria developed in the same way, generally speaking.

Independently,[10] I have found in diphtheria bacilli and diphtheroids the same structures as described by Löhnis and Smith, but I give them what I think is a simpler explanation in that I tried to explain all forms on the basis that the bacteria in their nature are Fungi imperfecti. This

[9] Jour. Agric. Research, 1916, 6, p. 675.

[10] Acta Oto-Laryngolica, 1918, 1, p. 131; Acta Medica Scandinavica, 1920, 53, p. 1.

explanation does not appeal to Löhnis and Smith; they state that de Negri [11] who studied a corynebacteria isolated from cases of malignant granuloma, concludes that it concerns blastomycetes, and then they go on to say: "Therefore he was carried away to the entirely incorrect conclusion that those large budding forms were some kind of 'blastomycetes' and the organism studied by him should be separated from the bacteria and placed among Fungi imperfecti. A comparative study of any of the common bacteria—for example B. subtilis—would easily have prevented this serious error." Both Almquist, as well as Löhnis and Smith, thus appear to be unwilling to draw any botanical consequences of their discoveries.

Now is the time to present the reasons in favor of the view that the bacteria really are Fungi imperfecti. In the first place, many bacteria in structure and form correspond fully with certain Fungi imperfecti. Take for an example of a hyphomycete a monilia; this may grow partly with hyphae, which form conidia by budding or separate into oidia, partly as a budding mycelium composed of long or short buds, a number of factors largely unknown being responsible for the particular forms that may predominate in a culture. Evidence that many bacteria grow in a similar manner can be obtained from many investigators. Personally I have studied, as stated, the diphtheria bacillus and found that it may appear in all the forms of the monilia, that it multiplies by budding, that it may give rise to long or short budding mycelium, that it grows in branching hyphae, which secondarily fall apart into oida, and that it may produce conidia. These studies were made on cultures of a single cell and the budding was observed in hanging preparations under the microscope. These cells in microscopic structure show a minute similarity, as Meyer particularly has demonstrated. Equally convincing appearances have been found in the case of other bacteria, for example, fig. 3 from Johan-Olsen illustrating B. mucoides and B. erythrosporus; fig. 4, from Bouel, DuJardin-Beaumetz, Jasnnet and Jouan demonstrating the microbe of peripneumonia, and finally figs. 2, 9, and 10 from Meirowsky [12] representing, respectively, the tubercle bacillus, Sp. pallida and B. paratyphosus B. In regard to Sp. pallida, Meirowsky states that the spirochetes, like tubercle and lepra bacilli, presumably are fragments of higher plants belonging possibly to the thread fungi. In a study of the corynebacteria, without being familiar with Meirowsky's investigations, I took the opposite view in that I tried to show that these bacteria could appear also in vibrio and spirochete form and concluded that the

[11] Untersuchungen zur Kenntniss der Corynebacterien etc.; Folia Micro-biol., 1916, 4 p. 119. Quoted by Löhnis and Smith.[9]

[12] Studien über die Fortpflanzung von Bakterien, Spirillen u. Spirochäten.

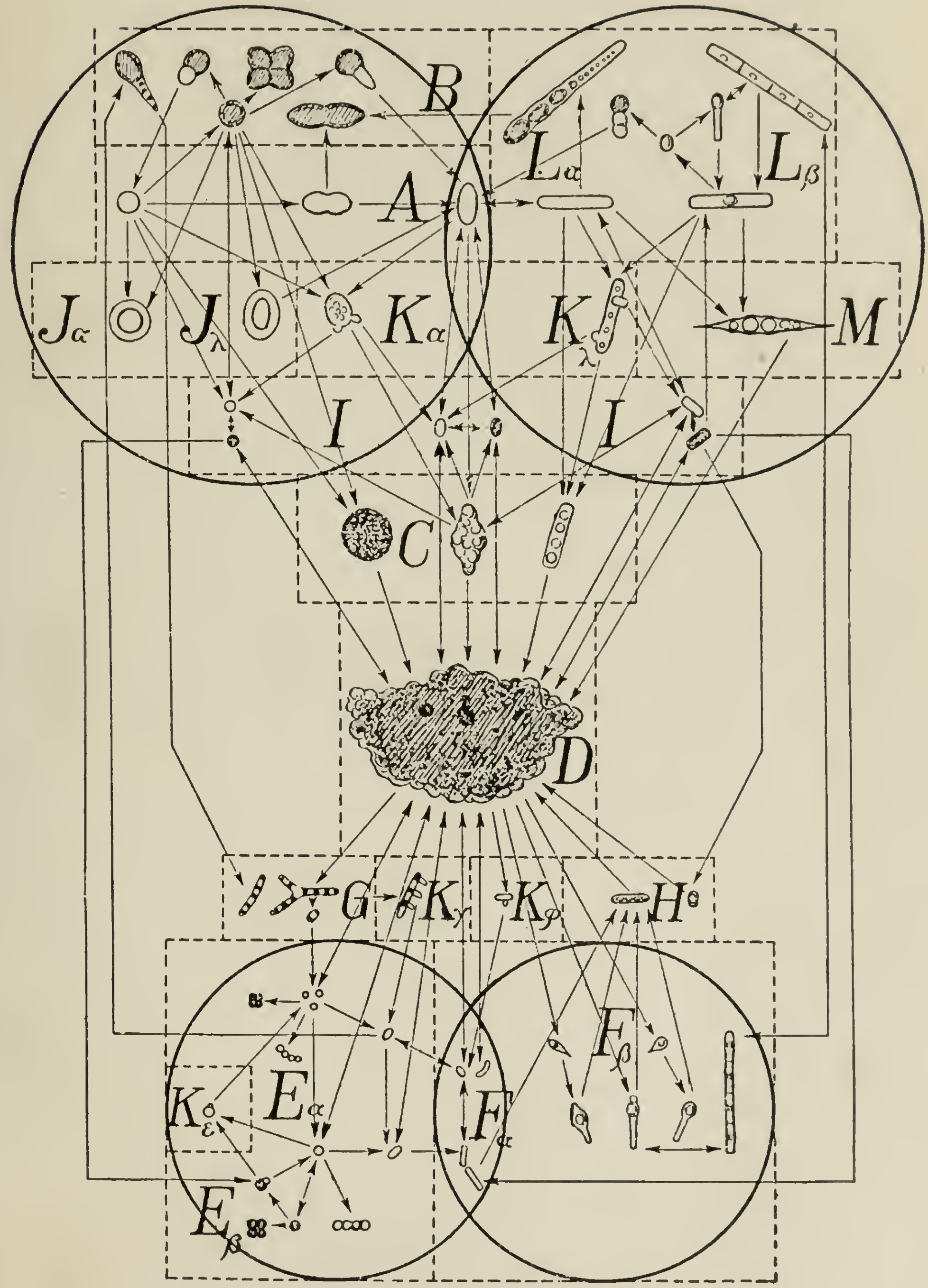
spirochete or spirilla and the fusiform bacilli in the so-called fusospirillary infections are simply developmental forms of the same organism. In this article I have brought together the views of recent investigators in regard to bacteria of different kinds in order to show that what is true in regard to the kinds just mentioned is generally true also.

In view of what has been said it would seem to be unnecessary to discuss the question of the relationship of bacteria with Flagellatae and Cyanophyceae. But the question of nuclei and spores, as well as motility of bacteria, deserves some consideration.

If the bacteria are Fungi imperfecti they should like these have a differentiated nucleus, and such it may be said has not yet been demonstrated. This is true, but as Lotsy states, it may not mean so much. Thus to demonstrate nuclei in objects as large as the yeast fungi has been associated with great technical difficulties and it may therefore be that the nucleus of the bacteria so far has escaped us. Besides, there are investigators who believe that they have demonstrated nuclei in bacteria, for example Nakanishi, A. Meyer and others. Of course if one regards bacteria as Fungi imperfecti one cannot accept the theory that the chromatin is spread diffusely in the cell body, because this assumes it would seem a much lower developmental stage.

As to the spore question, the endospores of the bacteria are lasting cells. Such are very frequent in the lower plants and animals, but according to many observers the spore-formation by bacteria is peculiar in that the protoplasm concentrates itself in one part of the cell and there surrounds itself with a membrane. According to Bütschli anything like this occurs only in a species of Flagellatae, a fact he cites in favor of his view that there is a close relation between the flagellates and the bacteria. The opposite of the formation of such endospores would be a cyst formation from swelling of the cell membrane and thickening. Van Tighen divides Schizophytes in those with cysts and those with endospores. Others compare the endospores with the chlamydospores of fungi, notably Brefeld, and so far as I can see this is warranted. Thus Lotsy describes the formation of chlamydospores in Mucor as follows: Here and there in the mycelium the protoplasm clumps itself and becomes surrounded with a membrane. The same thing occurs in Ustilago. As mentioned, Meyer does not approve of this view and regards the endospores as the homologue of asci of hemiasci. Meyer's theory will probably not be accepted by many. If the ascus is regarded, as it is by Lotsy, as a diplosporangium that arises through copulation, then this theory seems still more unwarranted. However,

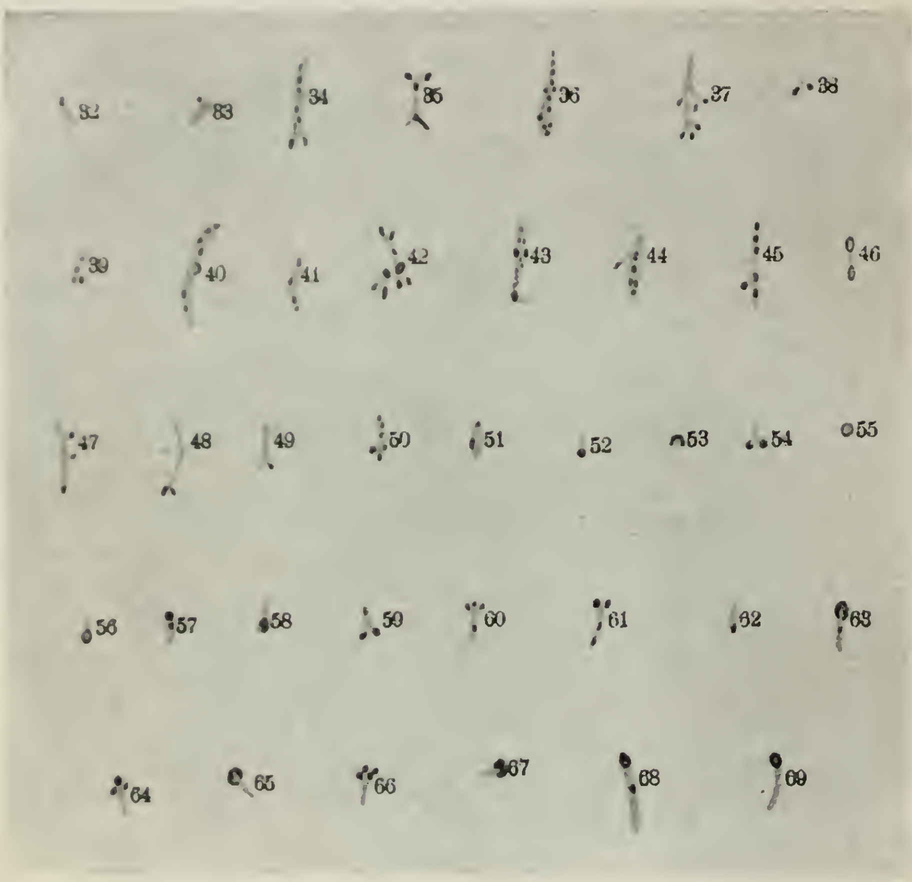
Plate 1



1

Fig. 1.—Life cycles of B. azotobacter after Löhnis and Smith. According to these authors, other bacteria, generally speaking, pass through similar cycles. It is of special interest to note the great similarity between the forms in the upper left circle and the diphtheria bacilli in plate 4, figure 6:28-36; figure 6:14 and plate 5, figure 8:7.

Plate II



2

Fig. 2.—B. tuberculosis, bovine type, according to Meirowsky.

3

Fig. 3.—B. mycoides (94) and B. erythrosporus (95) according to Johan Olsen.

PLATE III

4

Fig. 4.—The microbe of peripneumonia according to Bouel, Dujardin-Beaumetz, Jaennet and Jouan.

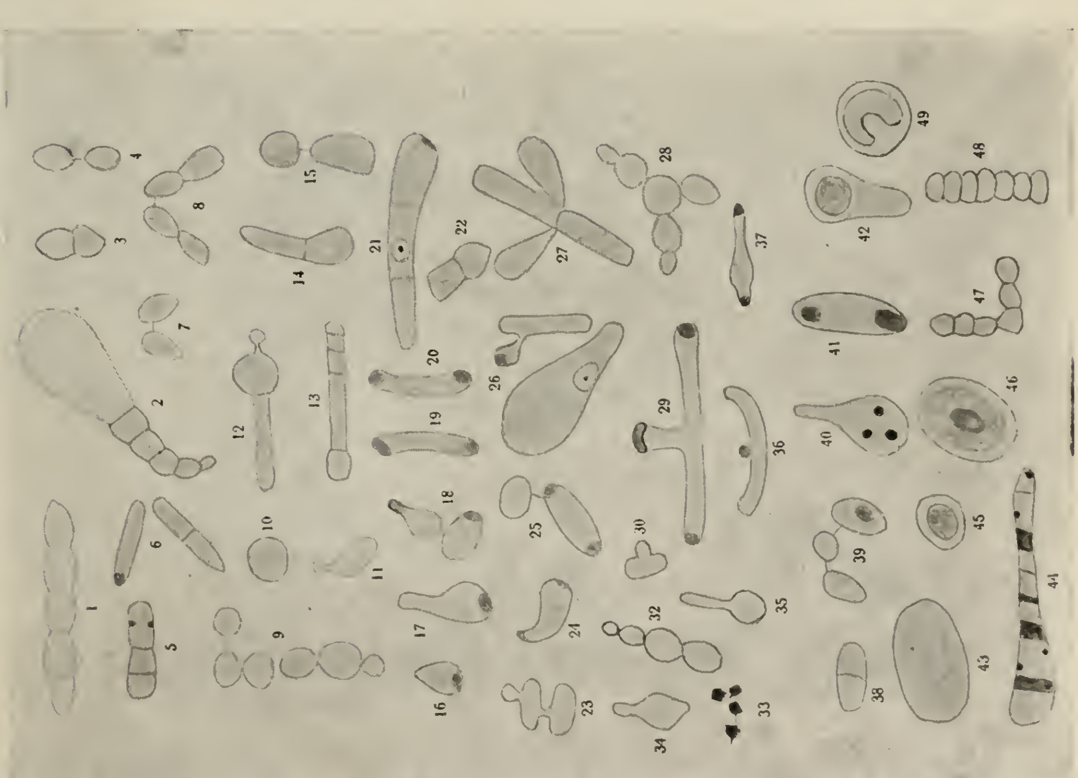


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Fig. 5.—B. diphtheriae according to Bergstrand.

PLATE IV

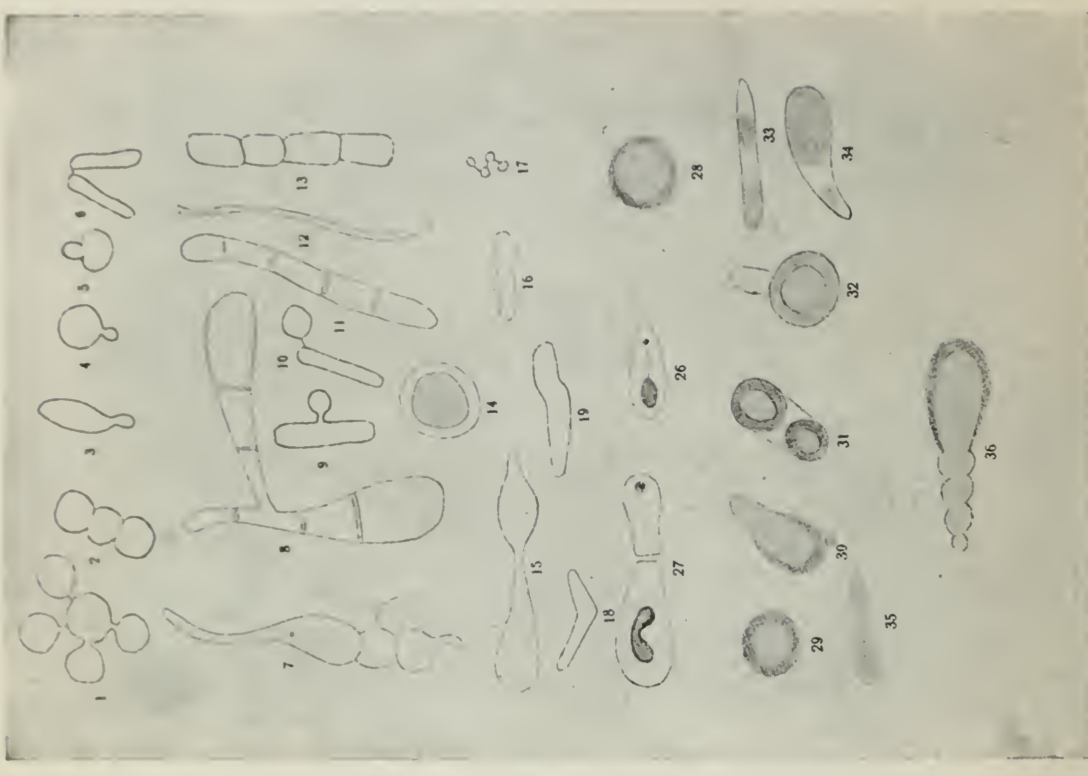

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Fig. 6.—B. diphtheriae according to Bergstrand.

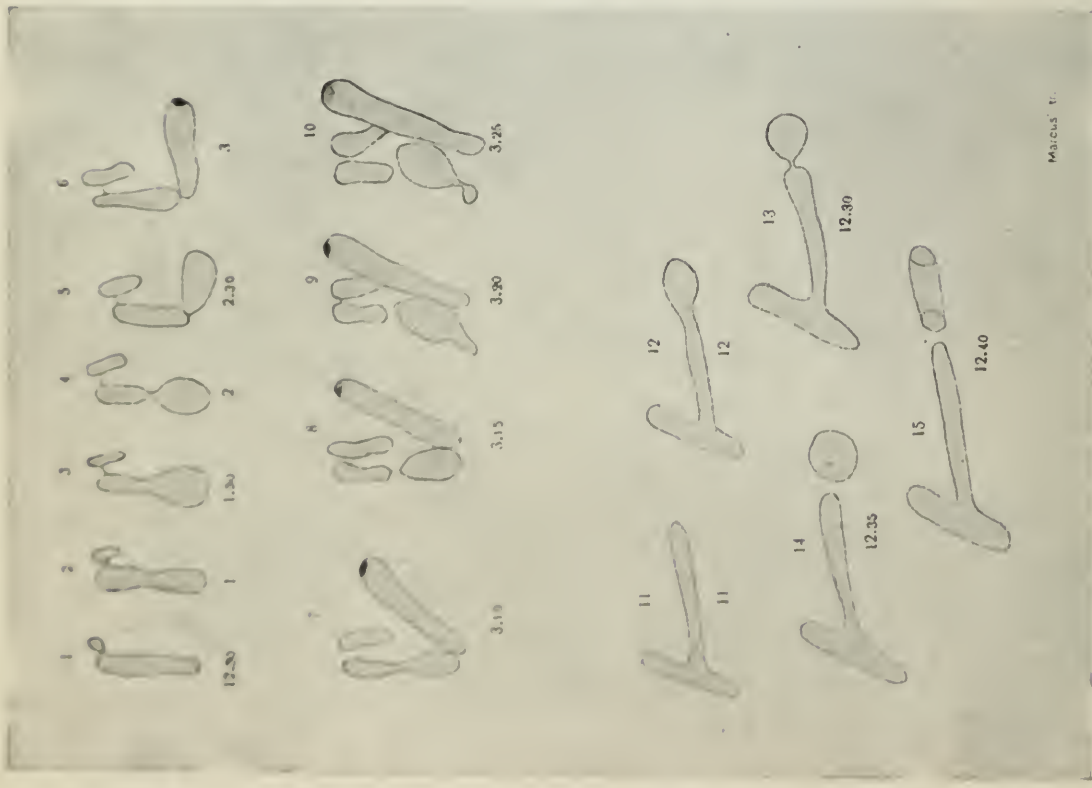

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Fig. 7.—B. diphtheriae according to Bergstrand. This illustration shows the development of this bacillus as followed under the microscope in hanging blocks.

Plate V

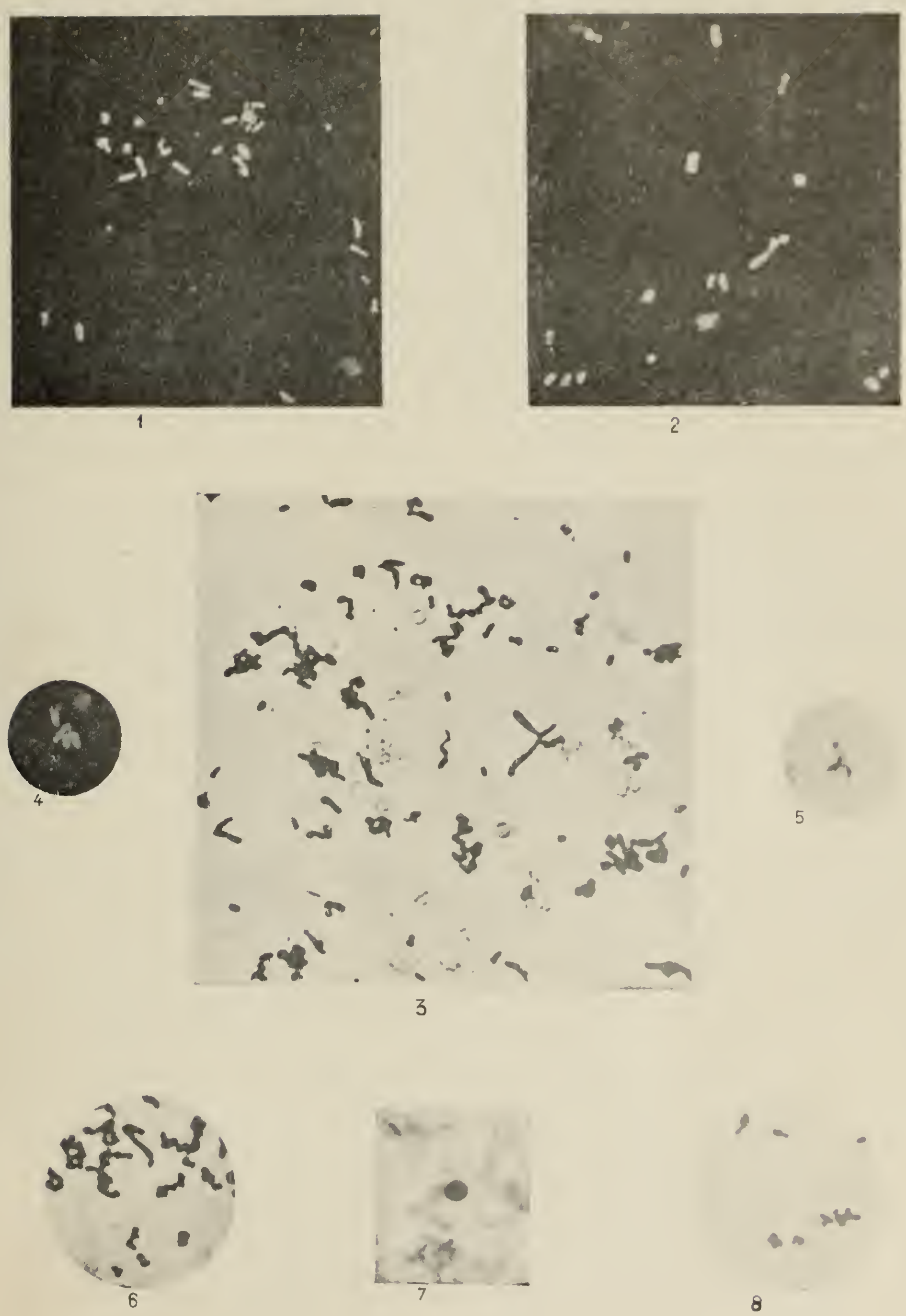

Fig. 8.—B. diphtheriae according to Bergstrand. Good budding forms are seen especially in 1 and 8; 3 shows a branching bacillus in different stages of separation into oidia; 7 illustrates a double contoured cyst-like lasting form.

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Fig. 9.—Spirochaeta pallida according to Meirowsky. Some forms show a definite budding, and round coccuslike forms are also seen.

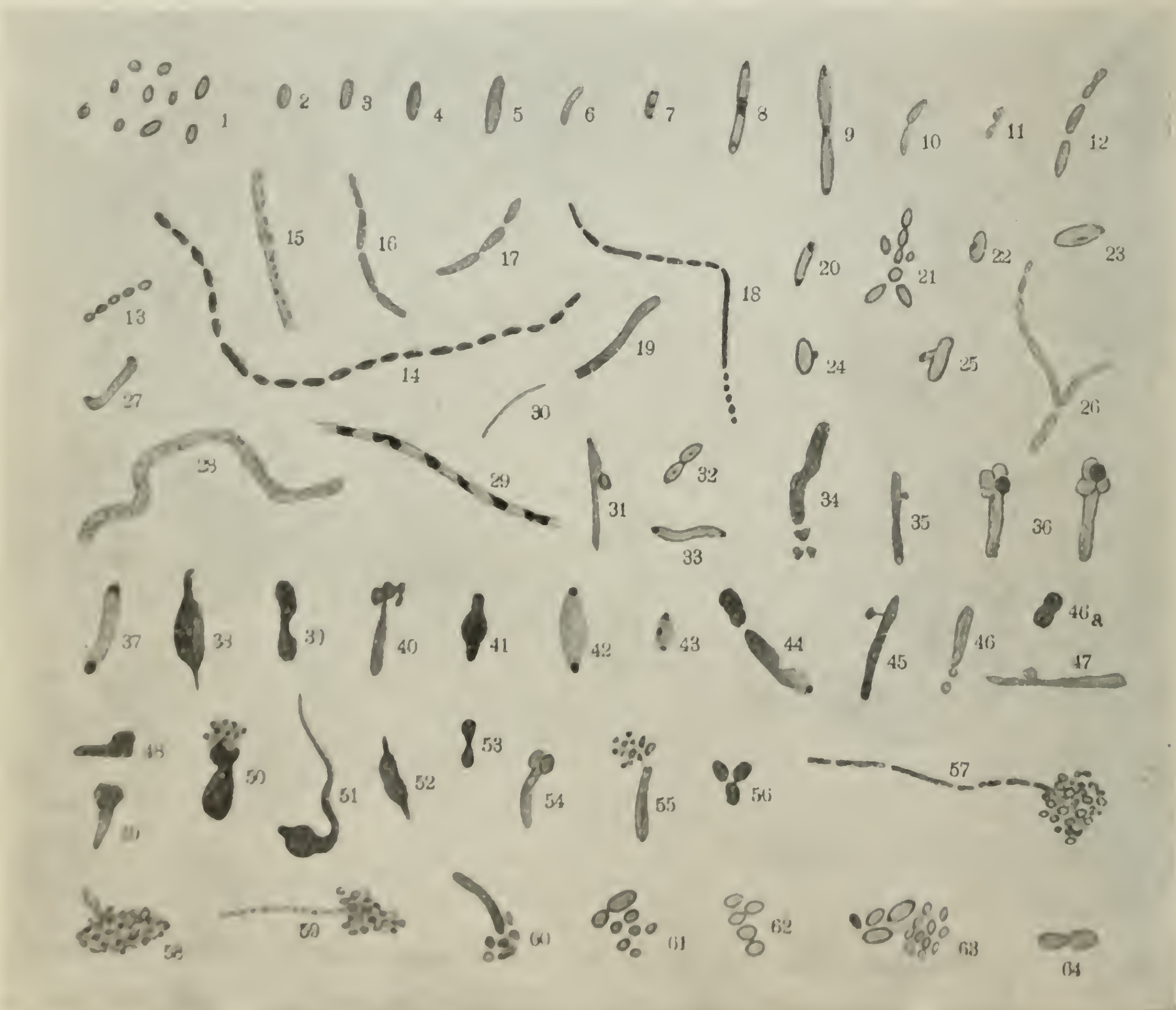

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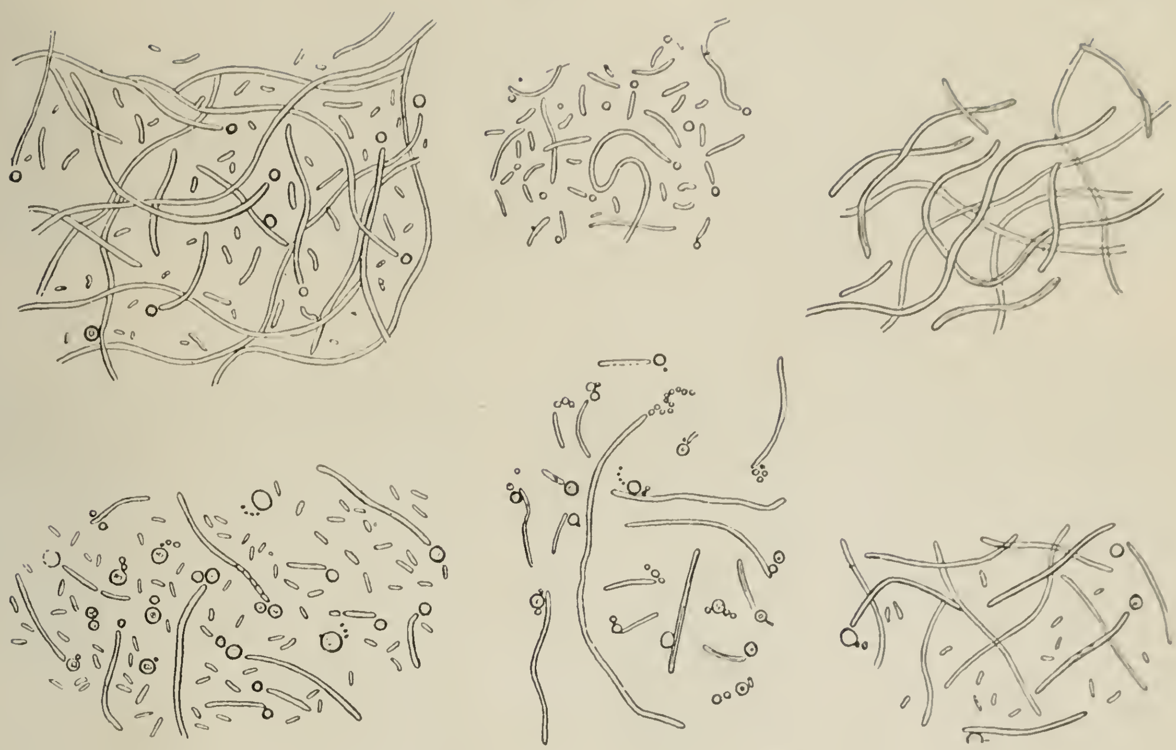
Fig. 10.—B. paratyphosus B according to Meirowsky. Note especially 29, which shows a long thread in which the protoplasm has formed clumps, which are more deeply stained, but the spaces between the clumps do not seem to have been empty. The significance of these forms, which are found in a variety of bacteria, has been discussed by Berg

PLATE VII



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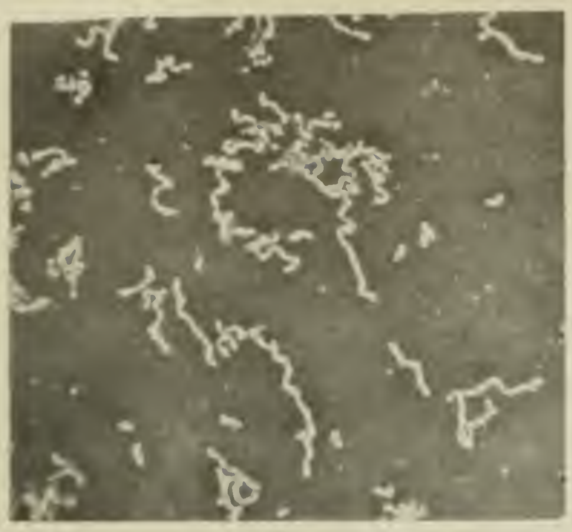
Figs. 11 and 12.—B. typhosus according to Almquist. Note the round forms, some of which are reproduced with thicker walls. Possibly it may concern structures similar to the double contoured bodies described by Löhnis and Smith and by Bergstrand. Note also the threadlike forms of the typhoid bacillus in figure 12, 9 (myceloid, Almquist).

Plate VIII



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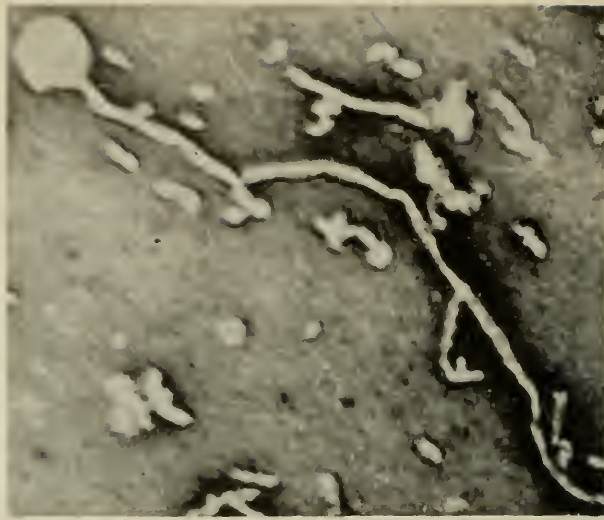

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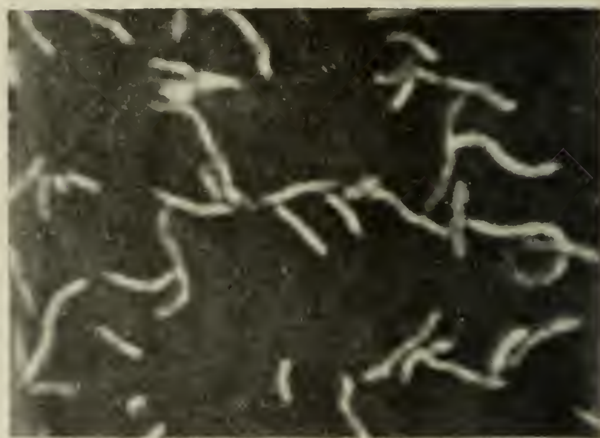

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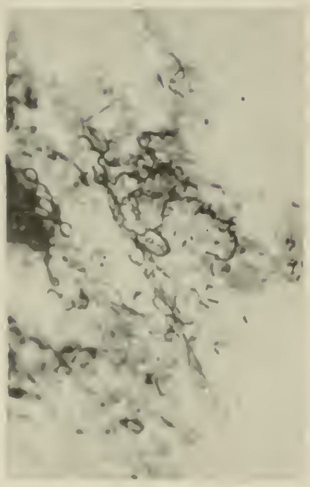

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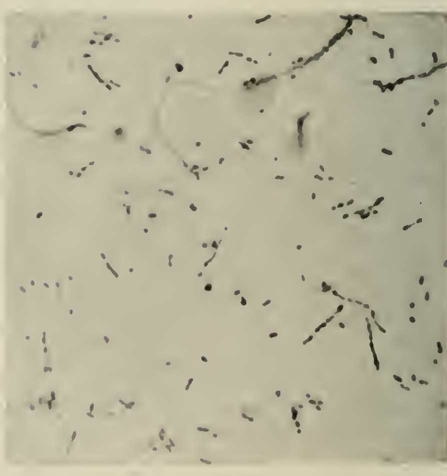

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Fig. 11. [illegible] Variable magnification. Note the marked polymorphism; even

this may be only apparent, and Lotsy regards it as possible that Saccharomycetes are reduced Ascomycetes that in part form diplosporangia by copulation but also haplosporangia parthogenetically. This opens the road for the bacteria. It seems to me that for the present it would be better to regard bacterial spores as vegetative; further reasons for this view will be given.

According to de Bary and Hueppe, bacteria have also another kind of spore, namely, arthrospores. These are rounded or oval structures that originate by construction of the protoplasm, the cell dividing itself, the daughter cells surrounding themselves with a thick membrane. These are, therefore, more like cysts. According to Hueppe, but scant attention has been given to arthospores. In diphtheria bacilli I have found two peculiar lasting forms, consisting of large acid-fast cells with thick double contoured walls, formed in the original cells, frequently one in each end of a rod. They do not deviate in any way from the chlamydospores of Mucoraceae. Frequently one finds only a small remnant of the original bacillus hanging to the spores. If this disappears we have a body, which greatly resembles cysts of Saccharomycetes. Figs. 6, 28 to 32 show all these forms, which clearly resemble the arthrospores of de Bary and Hueppe more than the endospores of the bacteria. Subsequently, I have observed similar bodies in many other bacteria and also the outgrowth of a rod-shaped structure through the thick membrane. Similar bodies are also found in Almquist's illustration (figs. 11 and 12), although he does not mention them. He mentions only rounded bodies that may grow out into rods, and that he calls bacterial conidia. Hueppe also discusses conidia, but neither explains what is meant by this expression. I think all these observations may be summarized as follows: Bacteria may grow as long and short buds; one form may develop from the other; from a coccus may grow a rod and vice versa; in addition there occur lasting forms with thick walls which likewise may develop from a coccus—or a rod-form. This does not occur by budding, but in the same way as endospores grow. Endospores and the structures described are related and probably homologous with the chlamydospores of fungi.

Finally, bacteria may develop in still another way: In the threads of actinomycetes arise as we know rounded or oval clumps of condensed protoplasm, which become more and more prominent at the same time as the thread otherwise becomes empty. The thread may burst and the clumps may be set free. Boström called these clumps spores and believed that they gave rise to new individuals. These structures are

found in most bacteria, if they are cultivated in the proper way. The indications are that they may reproduce their kind, as Boström believed, and this is not remarkable when we consider what has been said about certain algae in which a drop of protoplasm pushed out through a hole in the cell may give rise to a new individual.

It remains to consider the motility of bacteria, about which there is not much to say. This property is of great importance and the theories of Bütschli in regard to the relationship of the bacteria to Flagellatae may not be dismissed off-hand. However, if it is found that the immotile bacteria are fungi, it would seem that the motile also are fungi because it is hardly likely that motile and immotile bacteria have different phylogenesis. Mobility may be regarded as a property that bacteria regained when they as reduced higher fungi returned to living in water. The higher fungi are land inhabitants developed from aqueous plants a certain stage of which was a motile stage.

In his well-known work Lotsy quotes from Zopf at the end of his chapter on the bacteria to this effect:

> According to the theory of Cohn of the constancy of fission fungi it may be assumed that the forms discussed possess complete morphologic independence, that is to say, that under different nutritive conditions they reproduce always their own, consequently do not assume mutual genetic relations. For example, according to Cohn, a micrococcus form can produce only micrococci, not rods or spirals, and similarly spirals can give rise to spirals only and not to rods and cocci, etc.
>
> This theory now has only historical value. It has been replaced by the theory of Billroth and Nägeli, in regard to the genetic connection of fungi. As strengthened by the author (Zopf) this theory holds that the fission fungi, probably with some exceptions, are able to pass through different developmental stages corresponding to the vegetative forms already described. After the studies of Cienkowski on certain fission fungi and the investigation of Neelsen of the fungus of blue milk had shown the genetic connection of coccal, rod, and leptothrix forms, definite evidence was brought forward by Zopf that the highest developed fission fungi (Cladothrix, Begiatoa) form not only these developmental forms but also curved forms of all kinds (spirilla, spirochetes, vibrios, ophidomonades).

Lotsy comments as follows: We now know on the contrary that Cohn's theory has been completely verified and that Zopf's views have been found to be erroneous.

But as shown in my presentation, the conception swings back into harmony with Zopf's view in that the bacteria may be regarded as Fungi imperfecti developed through reduction of higher forms and not as lowly primordial organism to be placed at the very beginning of the organic world.

FURTHER OBSERVATIONS ON THE EFFECTS OF ROENTGENIZATION AND SPLENECTOMY ON ANTIBODY-PRODUCTION *

Ludvig Hektoen

From the John McCormick Institute for Infectious Diseases, Chicago

In previous papers [1] I have recorded observations which show that roentgenization of white rats, dogs and rabbits at about the same time as antigen is introduced may restrain greatly the production of antibodies as measured by the antibody content of the serum. I have also noted [2] that in dogs splenectomy just before the injection of foreign blood was followed by a lower but otherwise typical antibody curve than is usually the case in dogs under otherwise comparable conditions. In the meantime, additional observations have been made on roentgenization and splenectomy under more diversified conditions, the results of which seem to merit a brief report.

SPLENECTOMY

Experiments on white rats gave results similar to those in dogs. Without exception the amount of lysin for sheep corpuscles was much less in the rats in which the spleen was removed at the same time that the blood was injected. As seen on chart 1, the lysin curves present the same general outlines, but in the splenectomized series the latent period is longer, the height and duration less than in the controls.

Rats weighing from 70 to 80 gm. were used; 1 c c of a 10% suspension of sheep blood per kilo of weight was injected intramuscularly immediately after the splenectomy. The curves (chart 1) are composite curves based on the titers of two rats killed on each day indicated, it being practically impossible to bleed the same rat many times. The control curve gives the titer of rats of the same age and size, treated in the same way, but not splenectomized. The titer gives the highest dilution of the serum that caused distinct lysis in a mixture of 0.6 c c consisting of 0.2 c c of a 5% suspension of sheep corpuscles, well washed, 0.0125 c c of guinea-pig serum, and the indicated amount of heated rat serum, the rest being salt solution. The tubes were incubated for two hours and then placed in the icebox until the next morning.

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* This article with two additional tables is published in Contributions to Medical and Biological Research, dedicated to Sir William Osler, 1919, 2, p. 973.

[1] Jour. Infect. Dis., 1915, 17, p. 415; 1918, 22, p. 28.

[2] Ibid., 1909, 6, p. 78.

In rabbits splenectomy from one to six days before the intraperitoneal injection of 25 c c of sheep blood as a rule did not interfere with the production of lysin and precipitin; exceptionally, however, splenectomy shortly before the injection seemed to suspend completely the advent of antibodies into the blood.

Of 6 rabbits splenectomized 5-9 weeks before the injection, all save one developed about the usual amount of lysin and also precipitin in fairly high degree, though with a somewhat prolonged latency. Of 10 rabbits, all young and healthy, splenectomized from 5-9 weeks before the intraperitoneal injection of 30 c c of human

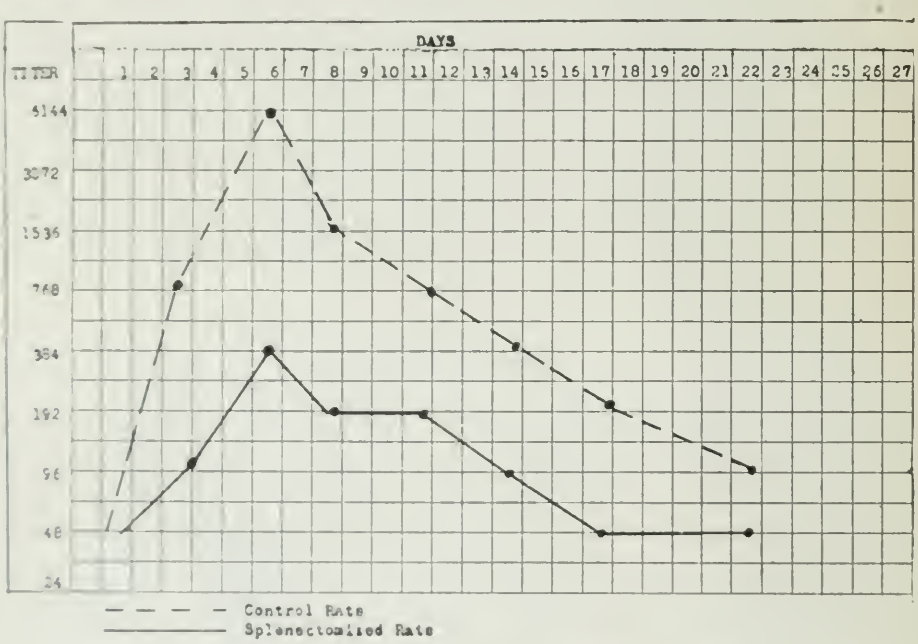

Chart I. Lysin in Normal and Splenectomized Rats.

blood, all but one failed to develop more than a trace of precipitin, but in all agglutinin of considerable strength developed as well as smaller amounts of lysin. The last result is of interest because it suggests that under certain conditions splenectomy, even some time before immunization, may restrain the formation of one kind of antibody more than others.

In the experiments 25 c c of sheep or human blood were injected intraperitoneally in one dose. The highest active dilution of the serum was determined in the case of the lysin and agglutinin tests, and the highest dilution of sheep or human blood in which the rabbit serum caused precipitate by the ring or contact method after 2 hours at the room temperature was determined in case of the precipitin tests. The lysin and agglutinin tests were carried out in mixtures of 0.6 c c containing 0.2 c c of a 5% suspension of washed corpuscles, heated rabbit serum, and in the lysin tests guinea-pig serum (complement), the

rest being salt solution. The complement dose was 0.006 c c in the tests for lysin or sheep corpuscles, and 0.02 c c in the tests for lysin for human corpuscles. All lysin and agglutinin mixtures were incubated for 2 hours and then placed in the icebox until the next morning.

Taken as a whole, my results correspond well enough with those of earlier observers, some of whom obtained inhibition of antibody-production from splenectomy (London,[3] Deutsch [4]), while others failed (Jakuschewitsch,[5] Kraus and Schiffman,[6] McGowan [7]), but minute comparisons are not worth while because of great differences in the experiments, e. g., mode of injection of antigen, measurements of antibodies, animals used, etc.

SPLENECTOMY AND ROENTGENIZATION

Table 1 gives details of an experiment on young dogs of the same litter in which roentgenization and splenectomy, alone and combined, greatly reduced the output of lysin after injection of goat blood. The small number of dogs represented precludes any conclusion as to which procedure may be most effective, but the results of splenectomy alone or combined with roentgen ray shortly before the antigen was injected seem the more striking.

In this, as well as in the other experiments discussed, the roentgenization was done in the Presbyterian Hospital by Earl Ball. The Coolidge tube was used, the focal distance was 8 inches, the current 5 to 6 milliamperes, spark-gap 8 inches. In the tables the dose is expressed in calculated Kienbach units. Usually two exposures were given, a major and one one-fourth as long the next day.

ROENTGENIZATION AND SPLENECTOMY AT HEIGHT OF ANTIBODY-PRODUCTION

Tables 2 and 3 and chart 2 give the results of new experiments [8] on the effect of the roentgen ray and splenectomy at or near the high point of the accumulation of antibody in the blood. These results indicate that neither roentgenization, as practiced, alone or combined with splenectomy, nor splenectomy alone or combined with roentgenization had any appreciable influence on the course and amount of antibodies in the blood when applied several days after the introduction of the antigen. The experiments covered by tables 2 and 3 concern in each case young dogs of one litter.

[3] Arch. d. Sc. biol., 1901, 8, p. 328.
[4] Ann. de l'Inst. Pasteur, 1899, 13, p. 688.
[5] Ztschr. f. Hyg. u. Infektionskr., 1904, 47, p. 407.
[6] Ann. de l'Inst. Pasteur, 1906, 20, p. 225.
[7] Jour. Path. and Bacteriol., 1911, 15, p. 262.
[8] Hektoen: Jour. Infect. Dis., 1918, 22, p. 28.

TABLE 1

ROENTGENIZATION (45 KIENBACH UNITS) AND SPLENECTOMY, SINGLY AND COMBINED, SHORTLY BEFORE AND 5 DAYS AFTER INJECTION OF GOAT BLOOD IN DOGS

Number of Days After Injection of Goat Blood	1 Roentgen Ray 2 Days Before Injection	2 Roentgen Ray 2 Days Before Injection and Splenectomy 5 Days After	3 and 4 Roentgen Ray 2 Days and Splenectomy 1 Day Before Injection		5 Splenectomy 1 Day Before Injection	6 Splenectomy 1 Day Before and Roentgen Ray 5 Days After Injection	7 Control
2	0	0	0	0	0	0	48
3	0	0	0	0	48	48	192
4	0	0	0	0	96	48	384
5	96	96	48	0	0	192	768
6	384	96	48	0	0	384	1,536
7	384	192	48	48	96	384	3,072
8	768	384	96	48	0	384	3,072
9	768	384	96	48	0	384	3.072
10							
11	192	96	48	48	192	192	1,536
12	192	96	48	48	0	192	768
13	192	96	48	48	0	192	384
14							
15	96	0	0	0	0	48	192
16							
17	96	0	0	0	0	96	192
18	96	0	0	0	0	48	192

TABLE 2

ROENTGEN RAY (45 KIENBACH UNITS) AND SPLENECTOMY, SINGLY OR COMBINED, IN DOGS AT OR NEAR HEIGHT OF PRODUCTION OF LYSIN FOR GOAT CORPUSCLES

Number of Days After Injection of Goat Blood	1 Roentgen Ray on 6th Day	2 Splenectomy on 6th Day	3 Splenectomy on 6th Day and Roentgen Ray on 7th Day		4 Control
3					
4	192	192	.....	.....	96
5	768	768	192	768	384
6	768	768	384	1,536	768
7	1,536	1,536	768	3,072	768
8	3,072	1,536	1,152	1,536	1,536
9	3,072	3,072	1,152	1,536	1,536
10	6,144	3,072	1,536	1,536	1,536
11	3,072	3.072	1,536	1,536	1,536
12	3,072	3,072	768	1,536	3,072
13	1,536	768	768	768	768
14	1,536	768	768	768	768
15	1,536	.....	384	768	
16	1,536	768	384	768	768
17	.....	.....	384	768	
18	1,536	768	.....	.....	768
19	.....	.....	192	384	
20	768	384	.....	.....	384
21	....	.....	192	192	
22					
23	.....	.....	192	192	384
24	768	384			
25					
26	[illegible]	[illegible]	192	192	192
27					
28	768	192	...	...	192

TABLE 3

ROENTGEN RAY (45 KIENBACH UNITS) AND SPLENECTOMY, SINGLY OR COMBINED, IN DOGS AT OR NEAR HEIGHT OF PRODUCTION OF AGGLUTININ FOR RAT CORPUSCLES

Number of Days After Injection of Goat Blood	1 Roentgen Ray on 6th Day	2 Splenectomy on 6th Day	3 and 4 Splenectomy on 6th Day and Roentgen Ray on 7th Day		5 Control
4	96	96	...	...	96
5	192	192	0	0	192
6	192	192	192	96	192
7	384	384	192	192	384
8	768	768	384	384	768
9	768	768	768	384	768
10	768	768	768	576	768
11	384	768	...	...	384
12	384	384	768	384	384
13	384	768	384	384	384
14	192	384	384	192	384
15	...	...	384	192	
16	384	384	...	...	192
17	...	...	384	192	
18	384	384	...	...	192
19	...	...	384	192	
20	192	384	...	...	192
21	...	...	384	96	
22					
23	...	...	192	96	
24	192	384	...	...	192
25					
26	...	...	96	96	
27					
28	192	384	...	...	192

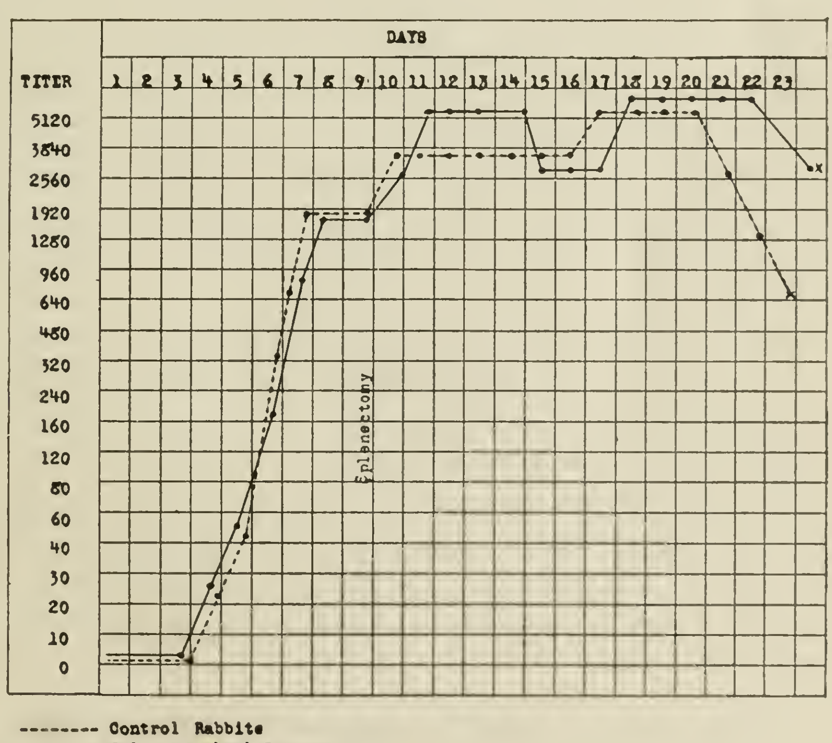

Chart II. Splenectomy at Height of Antibody Curve.

Beginning soon after splenectomy the red corpuscles were found more resistant to hypotonic solution than the corpuscles of nonsplenectomized animals. The increase in resistance seemed to be about the same in the splenectomized animals treated with roentgen ray as in those that were not; there was no change from the normal in the resistance of the corpuscles of animals subjected to the ray only. In the rabbits, too, splenectomy as a rule results in an increase.

Chart 2 illustrates the results of a study on 6 healthy young rabbits, each injected with 25 c c sheep blood, and in two of which splenectomy was made 9 and 10 days later, but without any effect whatever on the precipitin titer as compared with that in the controls.

These results are in full harmony with the results obtained by London [3] in an experiment on the effect of splenectomy some days after the production of hemolysin had started. I have reported previously that roentgenization of dogs when antibody-production is well under way has little or no effect on the antibodies in the blood.[8]

ROENTGENIZATION SIXTEEN DAYS BEFORE THE ANTIGEN IS INTRODUCED

Five young, healthy dogs of the same litter were given each an intravenous injection of rat blood; 16 days before 3 of the dogs had been roentgenized for 15 minutes and again for 3 minutes the day after (54 K. units). Two days before the injection of the rat blood the leukocytes ranged from 14,666 to 17,000 in the roentgenized dogs and in the two control dogs the counts were 11,333 and 15,666; the differential counts were normal. Table 4 shows that the agglutinin titer ran uniformly higher in the dogs treated with the roentgen ray than in the controls.

In another experiment 3 dogs were roentgenized for 20 minutes (60 K. units) and 15 days later injected with goat blood. These animals developed profound effects and died a few days after the injection without having produced hardly any lysin.

The first experiment indicates that under certain conditions the roentgen ray induces such changes in the body that the power to elaborate antibodies is increased. It lies near at hand to associate this increased power with regenerative changes in the lymphatic tissues and spleen after roentgenization.

In all the experiments on dogs, the antigen, 1 c c of 10% suspension of goat blood or rat blood per kilo of weight of dog, was injected intravenously. Only one injection was given. The figures in the tables give the highest active

dilutions of the dog serums in mixtures of 0.6 c c containing 0.2 c c of a 5% suspension of washed goat or rat corpuscles, the proper amount of dog serum, 0.0125 c c guinea-pig serum in the lysin tests, the rest being salt solution. The mixtures were incubated for 2 hours and kept in the icebox until the next morning.

TABLE 4

AGGLUTININ PRODUCTION IN DOG PREVIOUSLY ROENTGENIZED (54 KIENBACH UNITS)

Number of Days After Injection of Rat Blood	Roentgenization 16 Days Before Injection of Rat Blood and Again for 3 Minutes the Next Day			Control	
	1	2	3	1	2
3	48	24	24	96	48
4	384	192	384	96	192
5	1,536	192	384	384	192
6	6,144	768	1,536	768	192
7	6,144	768	3.072	1,536	384
8	3,072	1,536	6.144	1,536	384
9	6.144	3.072	3 072	1,536	768
10	3,072	3,076	1.536	1,536	1,536
11	3,072	3,076	3,072	768	1,536
12	1,536	1,536	3,072	768	386
13	1,768	1,536	1,536	768	384
14	354	768	384	384	192
16	192	384	192	192	192
18	192	384	192	192	192
22	192	384	96	192	48
25	96	192	45	96	96

SUMMARY

The results recorded show that splenectomy may diminish the output of antibodies especially when practiced about the same time the antigen is injected. In the rabbit, however, splenectomy under certain conditions may have little or no effect on antibody-production, as after a single large dose of sheep blood. On the other hand, even when made several weeks before injection of human blood, removal of the spleen seemed to interfere with the formation of precipitin, but further observations are needed to determine whether such selective effect occurs regularly under these circumstances.

On the whole, the effects of splenectomy at or near the time of injection of antigen appear variable and uncertain, more so perhaps than might be expected from the demonstrations that antibodies appear earlier in the spleen than in the blood,[9] that antigen is fixed by the spleen,[10] and that in the presence of antigenic substances cultures of splenic tissue outside the body may produce antibodies.[11] And yet

[9] Pfeiffer and Marx: Ztschr. f. Hyg. u. Infektionskranksr., 1898, 37, p. 272; Cantacuzene: Ann. de l'Inst. Pasteur, 1902, 16, p. 552; Tsurumi and Koda: Ztschr. f. Immunitätsf., O., 1913, 19, p. 519.

[10] Leuckart and Becht: Trans. Chicago Path. Soc., 1911, 8, p. 202.

[11] Carrell and Ingebrigtsen: Jour. Exper. Med., 1912, 15, p. 287.

variations in results are really not surprising if we consider, first, the close relation of the spleen to the lymphatic tissues and the marrow, which are believed also to take part in the elaboration of antibodies and consequently may be capable of compensatory activities, to say nothing of the possibilities of accessory spleens; and, secondly, that the experiments of different investigators were made under diverse conditions in such important respects as kind, quantity, and mode of injection of antigen, measurement of antibody, etc. Perhaps the effects of splenectomy would not be so variable in larger series of experiments with particular effort to secure as high a degree of constancy of the controllable factors as possible.

The results of several experiments indicate clearly that after antibody-production is well under way, splenectomy has little or no effect on the course of the antibodies in the blood. I have noted elsewhere [8] that the usual effects of the roentgen ray and of benzene appear to be withstood when antibody-production is well started. We now find that splenectomy, even when reenforced with roentgenization, seems to be subject to a similar resistance; at any rate, the antibody-content of the blood was not diminished markedly by splenectomy and roentgenization at or near the height of the curve. The nature of this so-called resistance remains obscure.

It may be pointed out again that as time passes after roentgenization the power to produce antibodies may be increased, and it is suggested that this increase may be due to regenerative changes in the spleen and lymph nodes. We consequently must distinguish between the immediate and the later effects of the roentgen ray. That the ray may reduce antibody-production seemed a good explanation of the increased susceptibility of guinea-pigs to tuberculosis described by Morton.[12] Kellert,[13] however, could not confirm Morton's claim; he found that roentgenization rather increased the resistance to the tubercle bacillus at the same time as the guinea-pigs seemed to become more susceptible to secondary and contaminating infections. Corper [14] also failed to produce any distinct effect on the gross tuberculous lesions in guinea-pigs by a single exposure to the roentgen ray. These contradictory results invite further experiments, not only on the effect of roentgenization on antibody-production, but also on phagocytosis and other cellular activities.

[12] Jour. Exper. Med., 1916, 24, p. 419.
[13] Jour. Med. Research, 1918, 39, p. 93.
[14] Am. Rev. of Tuberculosis, 1918, 2, p. 587.

DYSENTERY AND ALLIED BACILLI

Max Levine

From the Laboratory of the Central Medical Department, A. E. F., Dijon, France, and the Army Medical School, Washington, D. C.

In France we not infrequently experienced difficulties in growing dysentery bacilli and work was therefore begun (1) to differentiate the true dysentery bacilli, which are universally recognized as pathogenic, from the atypical or dysentery-like organisms (B. ambiguus, B. alkalescens, and B. dispar) many strains of which are nonpathogenic and whose etiologic significance is questionable; (2) to devise a more dependable, and if possible more simple medium, than the nutrient agar (phenolphthalein titration) for the isolation of dysentery bacilli.

The nomenclature in the group of dysentery bacilli has become quite confused. In this paper the following will be adhered to: B. dys. Shiga corresponds to the original Shiga-Kruse mannite negative type. The term B. flexneri includes both the B. dys. Flexner and Y types, and when possible it will be qualified with the race of the strain, such as V, W, X, Y or Z. The terms B. dys. Flexner and B. dys. Y are used in their old significance.

Serologic tests and studies on classification were beyond the scope of the investigation. Agglutination with stock Flexner and Y serums were carried out with 59 cultures. Acid production in a number of sugars and other fermentable substances, as well as the reactions in milk and the indol test, were observed on all the stains.

A total of 111 cultures were considered in this study. These were distributed as follows: B. dys. Shiga, 17; B. ambiguus, 5; B. flexneri, 60; B. alkalescens, 12; B. dispar, 11; miscellaneous, 6.

The Shiga cultures, with one exception, were stock strains found at the Central Medical Laboratory or the Army Medical School; several were duplicates.

The ambiguus strains included 3 (67, 68 and 69) from Dr. Andrews, St. Bartholomew's Hospital, London. One (4) was found at the Central Medical Laboratory marked B. dys. Shiga Fletcher vaccine stain, and another (101) obtained from the Army Medical School and probably a duplicate of (4), was marked B. dys. Shiga Fletcher 1. Serologic tests were not made with (101). The other (4) failed to agglutinate with several Shiga serums, and as both were positive for indol they are here considered as B. ambiguus.

The 60 cultures of B. flexneri include strains isolated during the war and also standard stock cultures. Included in this group are the old Flexner and Y types and authentic strains of the English groups V, W, X, Y, Z, VZ and WX, which were sent me by Dr. Andrews.

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There were 12 strains of B. alkalescens and 11 of B. dispar. These were received from Dr. Andrews or freshly isolated in laboratories of the A. E. F.

Of the 6 miscellaneous strains two (37 and 57) were marked B. ambiguus. They produced a green fluorescence in broth and on gelatin and were so different culturally from the strains of B. ambiguus received from Dr. Andrews that it seems they should not be considered as of the same group. Two cultures (48 and 108) were marked B. dys. Sonne. They did not agglutinate with the Flexner or Y serums available. Lactose was fermented with acid formation and then became alkaline. Milk was turned acid but not coagulated. These cultures resemble markedly some of the B. dispar of Andrews, at least culturally. One strain, 3, supposedly a Shiga, produced acid from sucrose and gave indol. It was not agglutinated with a Shiga serum. Another strain, 97, differed from all of the other cultures studied in that it fermented the glucoside salicin with a strong acidity in 24 hours.

AGGLUTINATION WITH FLEXNER AND Y SERUMS

Agglutination was made with living 24-hour broth cultures of 59 strains. The strains of B. dys. Shiga, B. alkalescens, and B. dispar were not agglutinated by either of the serums. B. dys. Sonne (48) and one of the English B. flexneri Z race (53), were also not agglutinated. It was noticed that the Z and X races of B. flexneri were only agglutinated in the low dilutions, and that (13 and 38) the original Mt. Desert Y and the Oxford Y strain, respectively, were not agglutinated even in 1 : 100 by the Y serum employed. From these observations it appears quite evident that what is regarded as the Y type of dysentery in different laboratories is not of the same serologic group.

BIOCHEMICAL REACTIONS (TABLE 1)

All strains were gram-negative short rods, and nonmotile as determined in semisolid agar (0.5% agar in broth).

TABLE 1

ACID PRODUCTION AND INDOL (PERCENTAGE OF POSITIVE REACTIONS) BY DYSENTERY AND CLOSELY ALLIED BACILLI

Organism	No. of Strains	Mannitol	Lactose	Glycerol*	Dextrin	Dulcitol	Sucrose	Xylose	Raffinose	Rhamnose	Indol
B. dys. Shiga	17	0.0	0.0	0.0	0.0	0 0	0.0	0.0	0 0	0 0	0.0
B. ambiguus	5	0.0	0.0	0.0	0.0	0 0	0.0	0.0	0.0	100.0	100.0
B. dys. flexneri†	59	100 0	0.0	0.0*	40.0	0.0	64.4	0.0	79.7	16.9	83.1
B. alkalescens	12	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	100.0
B. dispar	11	100.0	100.0	81.3	0.0	18.2	81.8	81.8	91.9	100.0	81.8

* Slight acidity in 5-7 days but more alkaline than P_H 7.0.
† Includes all mannite fermenting true dysentery bacilli.

Tests for acid production were made on glucose, mannitol, lactose, glycerol, sucrose, dextrin, arabinose, dulcitol, rhamnose, xylose, raffinose and salicin. The medium employed consisted of 1% peptone, and 0.4% dipotassium phosphate with 1% of the test material. The rosolic acid-china blue mixture of Bronfenbrenner was the indicator. Incubation was at the body temperature, and observations were made daily for 7 days.

The indol reaction was determined from peptone water after 5 days' incubation by the nitroso-indol reaction. Litmus milk was observed for 13 days

Table 1 indicates that the 5 main types of dysentery and dysentery-like organism may be readily differentiated by fermentation and indol reactions

Thus B. dys. Shiga and B. ambiguus may be distinguished from the others (B. flexneri, B. alkalescens, and B. dispar) by the inability of the former to give acid from the alcohol mannitol. They differ from each other in that B. ambiguus forms indol and ferments rhamnose.

B. flexneri may be differentiated in a large proportion of instances from B. alkalescens and B. dispar by the reaction in glycerol and xylose. None of the Flexner strains produced acid from xylose, whereas this substance was fermented vigorously by 21 of 23 strains of B. alkalescens and B. dispar. Differentiation by glycerol fermentation was not so distinct, as a number of the Flexner strains produced a small amount of acid. Quantitative studies showed that this acidity was never beyond the true neutral point P_H 7.0. in 5 days. With the indicator employed, however, the results might be confusing in inexperienced hands.

B. flexneri differs also from B. alkalescens and B. dispar in the milk reaction. The former produces a faint acidity in litmus milk, which reverts very slowly, if at all, to a neutral reaction in from 10-13 days. B. alkalescens, on the other hand, reverts relatively rapidly, from 4-8 days, to a distinct alkaline reaction, while B. dispar becomes progressively more acid, eventually coagulating the medium. Unfortunately the milk reaction has not given concordant results in the hands of different observers, many recording distinct alkalinity and others coagulating with true dysentery strains of B. flexneri type.

It remains to differentiate B. alkalescens from B. dispar. The milk reaction has been referred to. The objectionable features of this reaction are the variability of different batches of milk and slowness of the test. The lactose fermentation of B. dispar, although distinct, is often long-delayed. Table 1 shows that although there is some overlapping, the two organisms are markedly different when groups of characters rather than single reactions are considerel. Thus B. alkalescens does not form acid from lactose, sucrose or raffinose, but attacks dulcitol vigorously, while B. dispar rarely ferments dulcitol, but does form acid from lactose and most always from sucrose (81.8%) and raffinose (91.9%). B. alkalescens seems to be a very homogenous group. B. dispar probably consists of several varieties. The indol-negative, xylose-negative variety of B. dispar corresponds culturally to the strain isolated by Sonne in Denmark.

VARIETIES OF B. FLEXNERI

A number of subdivisions of the mannite fermenting dysentery strains on serologic and biochemical reactions have been proposed in the past. The probable untenability of B. dys. Y as distinguished from B. dys. Flexner has already been referred to. The differentiation of B. flexneri by the English War Committee as determined by careful absorption tests into V, W, X, Y and Z races appears much more acceptable and desirable.

The value of differentiation of this group on fermentation reactions has fallen into disrepute of late. Thus the fermentation of maltose, sucrose and dextrin, which were formerly emphasized as differentiating varieties of mannite fermenting dysentery strains, is about to be discarded. Maltose was not employed in this study as it was considered unreliable on account of the difficulty in obtaining a product entirely free from glucose, and the ease with which it decomposes on sterilization. Of the tests tried with 59 strains of B. Flexneri the following positive results were obtained with substances that might be of value for subdivision: sucrose, 64.4%; dextrin, 40%; rhamnose, 16.9%; raffinose, 79.7%; and indol, 83.1%. The correlation coefficients for each

pair of characters is given in table 2[1] which shows rhamnose correlates best with the other characters. Subdividing on rhamnose, two groups are obtained as follows:

	Strains	Percent Positive Sucrose	Dextrin	Raffinose	Indol
Rhamnose +	10	80	70	0	100
Rhamnose —	49	60	32	94	78

TABLE 2

CORRELATION COEFFICIENTS FOR FERMENTATIVE CHARACTERS

	Sucrose	Dextrin	Rhamnose	Raffinose	Indol
Sucrose	......	+0.62	+0.43	−0.06	+0.35
Dextrin	+0.62	......	+.065	—0.46	+0.50
Rhamnose	+0.43	+0.65	......	—1.00	+1.00
Raffinose	—0.06	—0.46	—1.00	......	—0.45
Indol	+0.35	+0.50	+1.00	—0.45	

Raffinose fermentation is particularly interesting. Of 30 strains in the rhamnose-negative subgroup which fermented sucrose, all attacked raffinose; but, of 8 sucrose fermenters in the rhamnose-positive subgroup none attacked the trisaccharid. The source of the 10 rhamnose fermenting strains was: Strain 26 was isolated at the Cent. Med. Dept. Lab. from a patient and diagnosed as probably B. dys. Y; (60 and 61) were isolated at Lab. 1, A. E. F., from a patient and carrier, respectively, and reported as B. dys. Flexner and B. dys. Y. and sent in for further identification. As the foregoing diagnoses were based merely on the two serums available—Flexner and Y—the designations should not be accepted as final. It would be desirable to know to which race of Flexner bacilli they belong. The remaining 7 strains were received from Dr. Andrews and Dr. Inman of London. One, 94, was labelled B. flexneri Y race which is a sort of composite of the V, W, X and Z races. The other 6 strains were all B. flexneri Z race. Thus there seems to be a correlation between rhamnose fermentation and the Z race of B. flexneri. If subdivision is to be made at all on fermentation reactions, then it appears that rhamnose would be the logical choice.

ACID PRODUCTION FROM GLUCOSE

In order to devise a medium for the differentiation of B. alkalèscens and B. dispar from the other dysentery or dysentery-like strains, the effects of various constituents of a selected medium on the rate of acid production and reversion were studied. Ten cultures, 2 B. dys. Shiga, 2 B. dys. Y, 2 B. dys. Flexner, 2 B. alkalescens, and 2 B. dispar were chosen for study.

Concentration of glucose.—The medium consisted merely of peptone (Difco) 1% dipotassium phosphate 0.4%, and glucose in varying amounts 0.0 to 0.5%, prepared in the following manner: To 1,000 c c of distilled water in a flask was added 10 gm. of peptone, 4 gm. of dipotassium phosphate, and the flask was then heated until the contents were dissolved (about 20-30 minutes). The medium was then filtered through paper and enough of a freshly prepared 10% glucose solution was added to give the desired concentration of the carbohydrate. The medium was tubed (about 20-25 c c) and sterilized in the autoclave 10 minutes at 10 pounds, after which it was incubated to eliminate unsterile tubes.

[1] See Levine, Jour. Infect. Dis., 1918, 3, p. 253.

Inoculation was made with 0.1 c c of a 24-hour broth culture with incubation at body temperature.

H-ion concentration was determined daily for 4 days by withdrawing 1 c c of the culture into 4 c c of neutral distilled water (P_H 7.0) in a clean, flat bottomed test tube, and after adding the required amount of an appropriate indicator, the color was matched with H-ion standards. Great difficulty was encountered in obtaining neutral distilled water in the laboratory in France. It was found, however, that the error introduced by the neutralization of ordinary distilled water with a small amount of sodium hydroxid was only about 0.1, which was within the limits of error in reading. Such neutralized water had to be freshly prepared and quickly utilized.

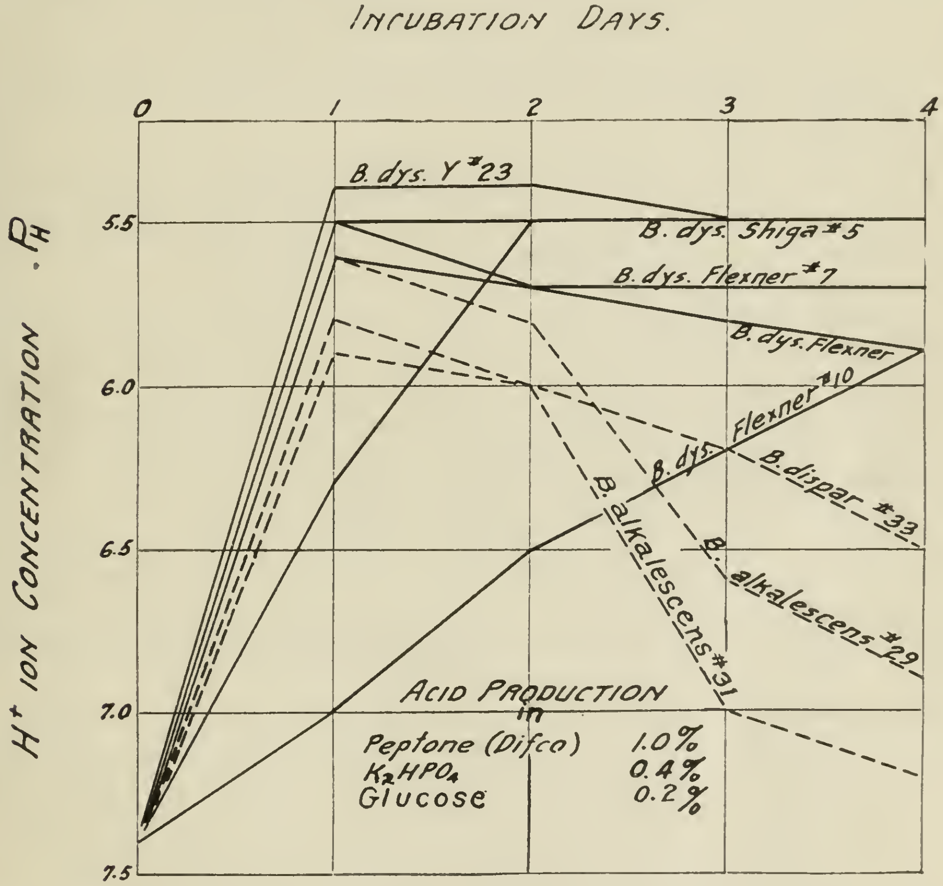

Chart 1.—Effects of peptone, dipotassium phosphate and glucose on the rate of acid production and reversion.

It was concluded (1) that with 1.0% peptone and 0.4% dipotassium phosphate, the employment of 0.3% or more glucose was undesirable for the purpose of differentiating B. alkalescens and B. dispar from the true dysentery bacilli; (2) that in the absence of glucose B. alkalescens and B. dispar form alkali more rapidly than the true dysentery strains (Shiga and B. flexneri); (3) that with 0.1% glucose there is reversion among the true dysentery strains, but B. alkalescens and B. dispar revert much more rapidly; (4) that with 0.2% glucose reversion among the true dysentery cultures was greatly inhibited, whereas B. alkalescens and B. dispar showed a marked alkali production after the primary acidity, as is shown in chart 1.

Concentration of Peptone.—The following experiment was made in Washington to determine the effect of the concentration of peptone on acid production and reversion:

Three batches of medium (0.2% glucose, 0.4% dipotassium phosphate, and 1.0%, 1.5% and 2.0% peptone respectively) were prepared as described; 7 c c portions were placed in tubes, autoclaved at 10 pounds for 10 minutes and incubated for 48 hours to eliminate unsterile tubes.

Seven tubes of each medium were inoculated from 24-hour cultures of organisms and incubated at 37 C.

Acidity determinations were made by the comparator in place of the dilution method previously described. Two duplicate cultures were taken and to one was added 0.3 c c of an appropriate indicator and the color matched with standards, the duplicate tube being employed to correct the error due to the color and turbidity of the culture medium in the comparator test. This tube was reincubatel and employed for this purpose in acidity determinations on subsequent days.

The concentration of peptone did not influence acidity production nor reversion of the true dysentery bacilli nor of B. ambiguus, but that with B. alkalescens and B. dispar reversion was much more rapid with 1.5% peptone than when 1.0% peptone was employed. Increasing the concentration to 2.0% did not further increase the rate of reversion.

Comparing the results with 1.0% peptone with those previously obtained in the original experiment in France, reversion was somewhat delayed in the new series. Although an adequate explanation is not available, it is felt that the difference is probably due to a difference in the actual concentration of glucose. The glucose available overseas was probably not thoroughly anhydrous.

Aeration seems to increase the rate of alkali production, after the primary acidity, in the case of B. alkalescens and B. dispar.

To determine whether the differentiation indicated in the quantitative observations could be applied qualitatively, each organism was inoculated in duplicate into the peptone phosphate medium containing as indicators 1% of a 0.5% phenol-red and 1% of a 0.2% brom-cresol-purple, respectively, and incubated at 37 C. Records of acidity were made daily.

With exception of (37 and 57), which have been referred to as probably misplaced in this group, and which remained alkaline throughout the experiment, all other cultures were distinctly acid to both indicators after 24 hours' incubation. With brom-cresol-purple as the indicator, all cultures of B. alkalescens and B. dispar showed reversion to distinct purple-blue color, as did also one of the B. dys. Sonne after 3 days' incubation. The true dysentery strains and B. ambiguus were yellow or brownish in color. On further incubation (6 days), the other strain of B. dys. Sonne and one B. flexneri became distinctly alkaline and a number of the true dysentery cultures began to show some reversion, thus obscuring, though not eliminating, the differentiation.

With phenol-red, on the other hand, all cultures of B. dys. Shiga and B. ambiguus, and all but one of B. flexneri were distinctly acid for 6 days. The 12 B. alkalescens strains were distinctly alkaline. Two of the 11 B. dispar were neutral, the others distinctly alkaline. One B. dys. Sonne was neutral and another alkaline

Rate of Acid Production.—It was observed that glucose was attacked more rapidly by B. alkalescens and B. dispar than by the other organisms of this collection Inoculation was from 24-hour broth cultures (0.1 c c to 30 c c of

medium) and H-ion determinations were made by the dilution method. In chart 2 the data are shown graphically.

The rate of acid production was observed qualitatively by the use of brom-cresol-purple and in some instances with the china-blue rosolic acid indicator. Inoculation was made from 24-hour agar slants; incubation was at 37 C. in the ordinary manner; acidity was recorded after 6 hours. At this time all strains of B. alkalescens and B. dispar, one B. flexneri and the 2 B. dys. Sonne were distinctly acid as indicated by a distinct or dirty yellow with brom-cresol-purple. All other strains produced acid less rapidly. They showed a distinct purple (more alkaline than P_H 6.3) with brom-cresol-purple and with the china-blue mixture were colorless or light blue.

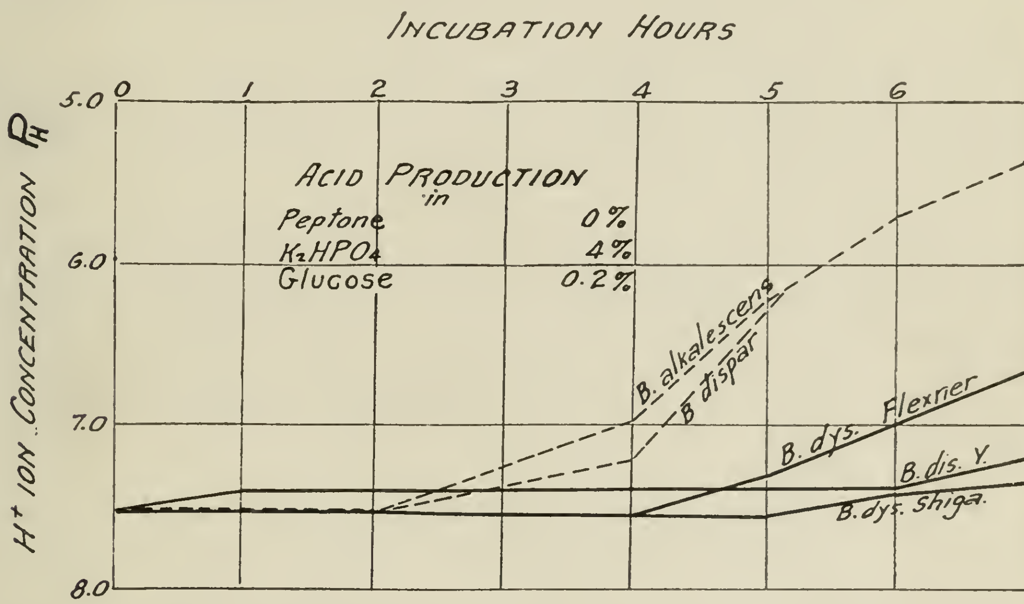

Chart 2.—Rate of acid production: Inoculations made from 24-hour broth cultures and H-ion determinations made by dilution method.

It may be concluded from the observations of glucose fermentation that B. alkalescens and B. dispar produce acid more rapidly and then revert to a distinctly alkaline reaction that may be indicated qualitatively by phenol-red or brom-cresol-purple. The use of brom-cresol-purple, however, would require experience and care, whereas phenol-red necessitates a prolonged period of incubation. The most desirable indicator for qualitative differentiation would be one which changes at P_H 6.5 showing a distinct coloration on the alkaline side.

A SIMPLIFIED SOLID MEDIUM FOR GROWTH AND ISOLATION OF DYSENTERY BACILLI

After a number of preliminary experiments it was found that by the addition of a small amount of glucose (0.03-0.05) to peptone-phosphate agar, growths as luxuriant, if not more so, than on nutrient agar could be obtained. As facilities for determination of the optimum H-ion concentration were not available at the time in France a series of experiments were carried out to determine the optimum concentration of dipotassium phosphate for growth of dysentery bacilli in a medium not requiring any further adjustment of reaction.

The medium consisted of 1.0% peptone, 0.1% glucose and 0.2 to 0.7% phosphate. Inoculation of the agar plates was made from a 24-hour broth culture. A concentration of 0.4-0.5% of the phosphate gave best results with 6 cultures examined. It is interesting in this connection to note that the titratable acidity with phenolphthalein was in each instance + 0.7%. The H-ion concentration was much more varied, probably 7.1 with the 0.2% of the phosphate and 7.8 with the 0.7% of the buffer salt, as indicated by subsequent experiments.

The influence of the H-ion concentration on the growth of the dysentery bacilli seems marked on solid medium. It has been my experience that in liquid medium the effect of the H-ion concentration is not so evident.

Experiments on the effect of the concentration of dipotassium phosphate repeated with 39 strains using 3 concentrations of the salt (0.2, 0.45 and 0.7%, respectively), showed that:

1. The phenolphthalein titration is a poor index of the true acidity of the medium. The variation in the titrable acidity was close to the limit of experimental error, whereas the difference in H-ion concentration with the different quantities of phosphate was marked and distinct.

2. The optimum reaction is not the same for all strains of dysentery. Two (5.1%) grew best with the largest quantity of phosphate, four (10.2%) with the least amount of phosphate and twelve (30.7%) show their optimum growth when 0.45% of dipotassium phosphate was used. Seventeen (43.6%) did equally well on all of the 3 mediums. Considering all cultures, we find 33 (84.7%) to have done as well or better with 0.45% phosphate than on either of the other concentrations of this salt. The H-ion concentration with 0.4% of the phosphate is generally 7.4 or 7.5. This quantity was selected as probably the most reliable and desirable.

Choice of Indicator.—A distinct and noninhibitory indicator is an important adjunct to the successful isolation of dysentery bacilli. It was hoped that the eosin and methylene-blue combination of Holt, Harris and Teague, which was found so valuable in water work, might be successfully employed, particularly as it was reputed to be noninhibitory. Thirty-nine strains of dysentery bacilli were inoculated on agar with and without the indicator from a 24-hour peptone phosphate culture.

The composition of the medium was:

Agar	1.5%
Peptone	1.0%
Dipotassium phosphate	0.4%
Glucose	0.1%
Indicator per 100 c c of above	
Eosin 2% yellowish aq	2.0 c c
Methylene-blue 0.5% aq	2.0 c c

(The P_H of this medium was 7.5).

B. dys. Shiga was markedly inhibited. A slight growth was observed on prolonged incubation (48-72 hours). Sixteen, or 50%, of the mannite fermenting dysentery strain were partially inhibited.

Of a number of indicators tried, the china-blue rosolic acid mixture was found to be the least inhibitory when working with pure cultures. Similar results were obtained with artificial suspensions of dysentery organisms in normal stools.

SUMMARY AND CONCLUSIONS

Observations made on 111 strains of dysentery and dysentery-like organisms indicate:

1. The strains of B. dysenteriae Y used in different laboratories are not of the same serologic group.

2. The main groups of the dysentery and closely allied bacilli, B. dys. Shiga, B. flexneri, B. ambiguus, B. alkalescens and B. dispar, are readily differentiated by fermentation reactions. B. dys. Sonne appears to be intermediate between B. dispar and B. flexneri.

3. Subdivision of B. flexneri on fermentation reactions is not advisable, but the flexneri Z race seems to be characterized by acid production from rhamnose. This character is also strikingly correlated with an inability to attack raffinose when sucrose is fermented.

4. B. alkalescens and B. dispar form acid from glucose rapidly in a medium containing 1.5% peptone, 0.4% dipotassium phosphate, and 0.2% glucose, then revert rapidly to an alkaline reaction. B. dys. Shiga, B. flexneri and B. ambiguus form acid less rapidly and remain permanently acid or revert slowly.

5. Dyes, such as eosin and methylene-blue, the fuchsin-sulphite indicator, and excess of rosolic acid or china-blue were found to inhibit many strains of dysentery, particularly the Shiga type.

6. The following medium is suggested for isolation work:

Distilled water	1,000 c c
Agar	15 gm.
Peptone	10 gm.
Dipotassium phosphate	4 gm.

To each 100 c c of the melted medium add before using:

Lactose, 20% solution	5.0 c c
Glucose, 5% solution	1.0 c c
Rosolic acid (1.0% in 90% alcohol)	1.0 c c
China-blue (0.5% in water)	1.0 c c

The H-ion concentration of this medium, which requires no adjustment of reaction and does not need to be filtered when used on plates, is 7.4 to 7.5.

ON THE PROTECTION AFFORDED TO RED CELLS AGAINST HEMOLYSIS BY EOSIN *

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In 1900 Raab [1] and von Tappeiner [2] found that if infusoria are placed in a very dilute solution of a fluorescent dye (acridin) and kept in the dark, the organisms are not injured, but on exposing them to diffuse sunlight, death results. The researches of von Tappeiner and Jodlbauer,[3] and others have esablished that the photodynamic effect, associated with fluorescent dyes, is toxic not only for unicellular organisms, but also for enzymes, bacterial toxins, immune bodies, blood cells and even for the higher animals. The effect obtained by placing the photosensitive organisms in the dye and keeping them in the dark for some time is no greater than when the dye is added just before the organisms are exposed to sunlight, and it is immaterial, for the purpose of laking red cells, whether the stain is within the cell or not. Fluorescence is necessary for photobiologic action but the quantitative effect is not proportional to the amount of fluorescence. It is necessary that the fluorescent solution be in intimate contact with the substance on which it is to act; absorption of fluorescent waves alone does not suffice to produce photodynamic effects. The rôle played by oxygen in this phenomenon is still a matter of dispute, but the evidence seems to indicate that its presence is necessary.

Fluorescence is not limited to certain dyestuffs but is a property of many compounds found both in animal and vegetable life and these share with the dyes the common property of sensitizing protoplasm for photodynamic action.

Busck [4] and later Sellards [5] found that addition of certain substances, such as blood serum and egg-white, to solutions of photo-

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[1] Ztschr. f. Biol., 1900, 39, p. 524; 1903, 44, p. 16.

[2] München. med. Wchnschr., 1900, 47, p. 5.

[3] Ibid., 1904, 51, p. 737; Deut. Arch. klin. Med., 1904, 80, p. 427; 1905, 85, p. 386; 1906, 86, p. 468 and p. 479; Kudo and Jodlbauer: Biochem. Ztschr., 1908, 13, p. 24; Harzbecker and Jodlbauer: ibid., 12, p. 306; Neuberg: ibid., 13, p. 305; von Tappeiner: Ergeb. der Physiol., 1909, 8, p. 698; Neuberg and Galambos: Biochem. Ztschr., 1914, 61, p. 315.

[4] Biochem. Ztschr., 1906, 1, p. 425.

[5] J. Med. Research, 1918, 38, p. 293.

biologic sensitizers results in decreasing or completely inhibiting toxic action, but no protection was afforded by the addition of glucose, starch or gelatin. This interesting observation appeared to us to demand further consideration, since there are definite chemical differences between the inhibiting and noninhibiting substances. The inhibitory effect cannot be attributed to the amphoteric character or the colloidal nature of the protective substances. There is a striking difference in the nature and content of the amino acids of gelatin and egg albumin. The former wholly lacks tyrosin, tryptophan and cystin, and contains only minimal amounts of alanin, serin, aspartic acid, phenylalanin and histidin.[6]

Former work [7] on the protection afforded to immune bodies against the toxic action of ultraviolet light showed that the aromatic amino acids have the power of absorbing these waves, and since action by light cannot take place without absorption, the presence of these amino acids in the protein molecule is essential for this reaction. In the present work we have sought to find a correlation between the protection afforded to red cells by certain proteins against lysis by eosin and the nature of the amino acids in the molecule and our results indicate that the presence of tyrosin or tryptophan determines ability to protect.

The experiments were carried out with eosin (Grübler's w. g.) and red blood cells were used to indicate toxic action; 0.5 c c of a 5% saline suspension of red cells (sheep or ox) was placed in each of a number of small test tubes and to each, 1 c c of a 1 : 10,000 eosin in salt solution was added. The substances to be tested for protective action were likewise made up in salt solution in the concentrations as given in the table and the reaction adjusted to P_H 7.5-8.0. The tubes containing the suspension of red cells, eosin and substance to be tested for inhibitory action and also a number of control tubes were placed in the sunlight for a period of 30 minutes and after exposure immediately placed in the ice chest. The tubes were inspected at the end of 3 hours and again after 18 hours to determine the amount of lysis. Only in those tubes which after 3 hours showed that some hemolysis had taken place, was a slight increase noted after 18 hours. A certain number of tubes similar to the above kept in the dark in

[6] Fischer, Levene and Aders: Ztschr. physiol. Chem., 1902, 35, p. 70; Levene and Beatty: Ztschr. physiol. Chem., 1906, 49, p. 252.

[7] Harris and Hoyt: Science, 1917, 46, p. 318; University of Cal. Pub. Path., 1919, 2, p. 245; Hill and Schmidt: J. Infect. Dis., 1919, 25, p. 335.

order to rule out the possibility of any unlooked for factor being concerned in the reaction.

From the table it will be noted that protein substances, other than egg-white and blood serum, namely, ovomucoid, casein, edestin, Witte's peptone and deutero-albumose, inhibit the lytic action of eosin on red cells. Casein lacks glycocoll, gelatin contains about 16%; apparently the presence or absence of this amino acid in the protein molecule is immaterial. The experiments with pure amino acids show definite results. Of those tested, glycocoll, leucin, aspartic acid, α and β alanin, glutamic acid, cystin, phenylalanin and taurin offer no protection while tyrosin and tryptophan are very effective. To a limited extent this coincides with results reported by Hill and Schmidt [7] on the protection afforded by amino acids to immune bodies against the toxic action of ultraviolet light. There is, however, a striking difference, illustrated in the instance of phenylalanin. The inability of this amino acid to protect against eosin hemolysis shows that the benzene ring alone is not the determining factor. The lack of protection shown by mandelic acid, hydrocinnamic acid and phenylpropionic acid supports this view. Tyrosin, despite its comparatitve insolubility (1:2,400), and tryptophan [8] offer marked protection to red cells. The hydroxyphenyl group in a molecule is one of the factors determining ability to protect. This is clearly shown by the fact that hydrocinnamic acid and mandelic acid offer no protection while both orthocumaric and orthohydrocumaric exhibit marked protective ability. Addition of di- and trioxybenzoic acid, resorcin, salicylic acid, pyrogallol or phenol to a mixture of red cells and eosin solution prevents hemolysis on exposure to light.

Since gelatin contains appreciable quantities of prolin (5%), arginin (7.6%), lysin (2.8%) and oxyprolin (6.4%), these amino acids appear to play no part in protecting red cells against the photodynamic effect of eosin. Valin, isoleucin, serin and histidin were not available and no results can be reported. In view of the structure of these amino acids, it appears probable that they are also without influence. The first three do not have a ring structure and histidin lacks the benzene ring. Little or no protection is afforded by inosite. It must be remembered that although there are six hydroxy groups in this molecule, the substance is a derivative of the reduced benzene ring, hexamethylene, and does not contain a hydroxyphenyl group.

[8] This preparation was recrystallized repeatedly and did not give the Millon test.

The protection afforded to red cells against eosin hemolysis is not due to the absorption of those light waves that cause eosin to fluoresce. It was found that tubes containing red blood cells and eosin surrounded by a solution of a "protective substance" are hemolyzed in the same time as those surrounded by a water jacket. Tubes containing cells, eosin and a protective substance fluoresce strongly when placed in sunlight; the function of the protective agent does not consist in the absorption of all of the visible fluorescent waves. We have confirmed previous observations that the eosin must be in intimate contact with red blood cells in order to obtain hemolysis. No hemolysis was observed when a suspension of red cells in a quartz test tube was placed in a solution of eosin and exposed to sunlight. Change of acidity is also not a factor concerned in the reaction since no inhibition of hemolysis was observed on addition of a neutral phosphate buffer mixture.

Since photochemical action is conditioned on absorption of light waves, it appears to us that the protection afforded by certain substances is connected with their ability to absorb the active rays and thus prevent their absorption by the red blood cells. If this hypothesis is correct, the toxic action of eosin on protoplasm is determined by the presence of tyrosin or tryptophan in the protein molecules of the cell.

Although our results have shown that certain relations exist between the structure of the protein molecule and ability to protect against the photodynamic effect of eosin, they must nevertheless be considered largely of a qualitative nature. Although certain substances protect red cells against eosin hemolysis, the action is largely dependent on the concentration of eosin, time of exposure and concentration of the protective substance. A number of experiments were also carried out with rose bengal (1:200,000) as photosensitizer. The results obtained were less pronounced than in the eosin series, due no doubt, to the greater potency of this dye in causing hemolysis.

It may be of interest to mention that Bovie [9] has recently found that in a solution of eosin which had been in contact with thymol for some time and exposed to diffuse daylight, a precipitate in the form of a surface film appeared, indicating that a reaction had taken place between these substances.

[9] Chem. Engineer, 1919, 27, p. 141.

THE EFFECT OF THE ADDITION OF CERTAIN SUBSTANCES ON THE HEMOLYSIS OF RED CELLS BY EOSIN

Substance Added	Formula	Concentration	Result on Hemolysis
(a) Proteins:			
Gelatin		1%	No inhibition
Blood serum		1:5	Complete inhibition
Casein		1%	Complete inhibition
Edestin		1%	Complete inhibition
Witte's peptone		1%	Complete inhibition
Ovomucoid		1%	Complete inhibition
Deuteroalbumose		1%	Complete inhibition
(b) Amino acids:			
Glycocoll	$CH_2NH_2.COOH$	1%	No inhibition
Leucin	$(CH_3)_2:CH.CH_2.CH:(NH_2).COOH$	1%	No inhibition
Alanin	$CH_3.CH(NH_2).COOH$	1%	No inhibition
Alanin	$NH_2CH_2.CH_2.COOH$	1%	No inhibition
Aspartic acid	$HOOC.CH_2.CH(NH_2).COOH$	1%	No inhibition
Glutamic acid	$HOOC.CH_2.CH_2.CH(NH_2).COOH$	1%	No inhibition
Taurin	$NH_2.CH_2.CH_2.SO_3H$	1%	No inhibition
Cystin	$\{HOOC.CH(NH_2)CH_2.S-$ $\{S.CH_2.(NH_2)CH.COOH$	Saturated solution	No inhibition
Phenylalanin	$C_6H_5.CH_2.CH(NH_2).COOH$	1%	No inhibition
Tyrosin	$OH.C_6H_4.CH_2.CH(NH_2).COOH$	Saturated solution	Complete inhibition
Tryptophan (3 spec.)	$C_8H_6N.CH_2.CN(NH_2).COOH$	M/40	Complete inhibition
(c) Other substances:			
Mandelic acid	$C_6H_5.CHOH.COOH$	1%	No inhibition
Hydrocinnamic acid	$C_6H_5.CH_2.CH_2.COOH$	1%	No inhibition
Phenlypropionic acid	$C_6H_5.C \vdots C.COOH$	1%	No inhibition
Inosite	$C_6H_6(OH)_6$	M/40	Slight inhibition
		M/200	No inhibition
Ortho-cumaric acid	$OH.C_6H_4.CH.CH.COOH$	M/40	Complete inhibition
		M/200	Partial hemolysis
Ortho-hydrocumaric acid	$OH.C_6H_4.CH_2.CH_2.COOH$	M/40	Partial hemolysis
Gallic acid	$C_6H_2.(OH)_3.COOH$ 3:4:5	M/40	Complete inhibition
		M/200	Complete inhibition
Pyrogallol	$C_6H_3.(OH)_3$ 1:2:3	M/200	Complete inhibition
		M/1000	Partial hemolysis
Trioxybenzoic acid	$C_6H_2(OH)_3.COOH$ 2:3:4	M/200	Complete inhibition
		M/1000	Partial hemolysis
Dioxybenzoic acid	$C_6H_3.(OH)_2COOH$ 2 : 5	M/40	Complete inhibition
		M/200	Partial hemolysis
Thymol	$C_6H_3(CH_3).OH.CH(CH_3)_2$ 1:3:4	Saturated solution	Partial hemolysis
Phenol	$C_6H_5.OH$	M/40	Partial hemolysis
Sodium benzoate	$C_6H_5.COONa$	1%	No inhibition
Salol	$C_6H_4.(OH)COOC_6H_5$ 1:2	Saturated solution 1:1000	No inhibition
Glucose	$OHCH_2.(CHOH)_4.CHO$	1%	No inhibition
Cholesterol	$C_{27}H_{45}OH$	0.3% suspension in 1% gelatin	No inhibition
Resorcin	$C_6H_4.(OH)_2$ 1:3	M/200	Complete inhibition
		M/1000	Partial hemolysis
Salicylic acid	$C_6H_4.(OH).COOH$	M/40	Partial hemolysis

SUMMARY

The object of the investigation was to determine the cause of the inability of gelatin to protect red cells against the photodynamic action of eosin. Since gelatin lacks certain amino acids that are contained in the protein molecules which afford protection, experiments were

carried out to determine the protection afforded by individual amino acids.

It was found that tyrosin and tryptophan offer marked protection, while phenylalanin, glycocoll, leucin, aspartic acid, alanin, cystin and glutamic acid are ineffective. Certain other substances containing a hydroxyphenyl group in the molecule also protect red blood cells against hemolysis by eosin.

Since action by light cannot take place without absorption, it is possible that the protection afforded to red cells by certain substances against the photodynamic effect of eosin is due to the absorption of the active rays by the protective substance.

IRREGULAR TYPHOID STRAINS AND THE INFECTIONS CAUSED BY THEM

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In a review recently written by V. C. Vaughan [1] on a report published from the Public Health Laboratories of Cairo, Egypt, entitled "The Bacteriologic Examination of Suspected Typhoids," the following statement is made:

"It is possible and indeed highly probable that so far as vaccination has failed it is due to the disease being caused by other members of the typhoid group, which in all probability is much larger than we now appreciate. A second most interesting point brought out in this valuable report is that of the introduction of a disease into a country where it has not hitherto prevailed and the possibility of the newly introduced organisms supplanting kindred organisms already native to the country. There are many reasons for believing that in the various camps in this country different organisms became predominant and the dominating organisms changed from time to time with new importations. It is possible that the great differences in the death rates in the various camps in this country may have been due to this or similar causes."

The observations reported in this paper lend some support to the suggestion mentioned, namely, that the failures of antityphoid-paratyphoid vaccination may be the result of other members of the typhoid group or, as it appears in our case, to be variants of typical B. typhosus. Minor variations among strains of the same species of bacteria are not uncommon, but in rare instances it has been possible to detect some of the factors that induce the production of such variants. With the creation of a large stratum of population highly protected by vaccination against typhoid and paratyphoid fever, the appearance of isolated cases or of small group epidemics in the supposedly immune must suggest an explanation similar to the one offered in the review. Unfortunately, the analysis of the various strains of B. typhosus isolated from vaccinated typhoid fever cases are few, the available data are unreliable and mostly obtained by incomplete biochemical and

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[1] Jour. Lab. & Clin. Med 1919, 4, p. 645.

serologic methods. In fact, the recent findings of F. Mock [2] in 45 positive typhoid and paratyphoid fever cases at Mesves Hospital in France, representing cultures from vaccinated soldiers, emphasize this statement in every respect. It is not surprising to find in his paper, dealing with variants and irregular strains of the typhoid and paratyphoid group of bacteria, the conclusion that "these atypical paratyphoid organisms probably are involution forms of the true typhoid and paratyphoid bacteria." Some of his strains changed their cultural while others exhibited irregularities of their agglutination characteristics. It is therefore not unlikely that a more careful analysis of these strains would have forced a modification of the sweeping conclusions and would have led to a consideration of the epidemiologic importance of such atypical strains.

A review of the literature indicates that irregular typhoid and paratyphoid strains have been repeatedly reported.[3] Recent publications also call attention to the existence of nongas-producing paratyphoid and B. enteritidis strains, which may be readily mistaken for true B. typhosus, when only abbreviated methods for identification are employed.[4] In this connection we recall the conclusions of Tenbroeck [5] in his paper on a nongas-producing hog cholera bacillus in which the statement is made that his strain resembles in many respects B. typhosus, and it may be that some of the so-called typhoid cultures that are not agglutinated by antityphoid serum are nongas-producing paratyphoids. A detailed consideration of the other publications will be given in connection with the discussion of our own results.

Even since 1913, when one of us (K. F. M.)[6] analyzed rather superficially an irregular strain of B. typhosus, which had been isolated from a vaccinated army officer, we have studied several hundred typhoid and paratyphoid cultures isolated from man or after prolonged sojourn in the tissues of laboratory animals. Only recently two irreg-

[2] Jour. Lab. & Clin. Med. 1919, 5, p. 54.

[3] See the publications of: LeCount and Kirby: Trans. Chicago Path. Soc., 1903-1904, 6, p. 209; Faroy: Compt. rend. Soc. de biol., 1908, 64, p. 1093; Lafforgue. ibid., 1908, 65, p. 109; Marotte: Progrès méd., 1909, No. 28, p. 358; Babes and Feodorasco: Compt. rend. Soc. de biol., 1909, 66, p. 787; Fromme: Centralbl. f. Bakteriol., 1911, 58, p. 445; Goebel: ibid., 1914-15, 75, p. 376; Niolle, Raphael et Debains: An. de l'Inst. Pasteur, 1917, 31, pp. 373, 388 and 403; Raynaud and Négre: Compt. rend. Soc. de biol., 1912, 72, p. 534; MacAdam: Jour. Roy. Army Med. Corps, 1919, 33, p. 140.

[4] Messerschmidt: Centralbl. f. Bakteriol., 1912, 66, p. 35; Oette: ibid., 1913, 68, p. 1; Wagner: Ibid., 1913, 71, p. 25; Ohno: ibid., 1915, 75, p. 288; and Morse and Tyron: Boston Med. & Surg. Jour., 1917, 177, pp. 173, 216 and 255; Broughton-Alcock: Lancet, 1919, 2, p. 1023.

[5] Jour. Exper. Med. 1916, 24, p. 213.

[6] See footnote 2 in Riesman: Jour. Am. Med. Assn., 1913, 61, p. 2205.

ular typhoid strains isolated from two vaccinated laboratory workers attracted our attention. The epidemiologic circumstances leading to the infection and the interpretation which our observations suggest justify in our opinion a detailed account of the clinical and bacteriologic observations. The evidence to be presented also indicates that one of the irregular strains reverted to its typical ancestor by passing through an aged nonimmunized man. The history of the infections and the bacterial findings are:

Case 1.—A janitor in our animal house, aged 59, complained of chilly sensations, weakness and severe frontal headache on April 1, 1919. He had a temperature of 38.8 C. and was therefore admitted to the hospital. There was nothing noteworthy in his family history. About March 10, 1919, he had felt chilly and feverish for two or three evenings in succession, but in the mornings he was always able to attend to his duties. However, during the day he perspired freely and was more readily fatigued in carrying his usual load of feed and distilled water, etc., upstairs. On account of the severe illness and the death of his wife he had very little rest during the months of January and February. In July, 1917, he was given three injections of fresh army T. A. B. vaccine; each injection was followed by a moderate local reaction. He has had for the last two years intimate contact with rabbits, guinea-pigs, cats, dogs and goats that discharged living typhoid bacilli. The two months preceding his illness about 30 to 40 rabbits, either renal or gallbladder typhoid bacilli carriers, and numerous guinea-pigs infected spontaneously with rodent paratyphoid B. bacilli and B. enteritidis were kept in the section of the animal house under his supervision. The same caretaker handdles also the cremation of these animals, which are always carefully wrapped by the experimenter in heavy paper. He never took any meals in the animal house and used water, soap and cresol solutions before returning to his home. However, in his spare moments he smoked cigarets, which he rolled himself.

Physical Examination.—The patient was well nourished and well developed; aside from his flushed cheeks he did not look very ill. There were no rose spots and no glandular enlargements. Liver and spleen were not felt. Both lungs showed normal dulness and breath sounds. The urine showed no albumin, sugar or sediment; the diazo reaction was negative. The blood count was 4,300,000 and 7,200 with 65% neutrophils and 24% lymphocytes. Temperature was 38.6 degrees, pulse 90, and respiration 26.

From the blood cultures (5 c c of blood in 200 c c of glucose broth and 2 c c in 10 c c glycerin peptone ox bile) (taken in the afternoon of April 1, 1919) a gram-negative typhoid-like organism grew in 18 hours. The agglutination test made on the same day was:

B. typhosus formalinized antigen, 1:10, + + +.

B. typhosus living polyvalent, 1:40, + +.

B. typhosus paratyphosus A, formalinized, 0.

B. typhosus paratyphosus B, formalinized, 0.

On the third day of the patient's stay in the hospital, during which time the temperature did not rise over 38.2 C., it fell to 37 C. and remained normal. On April 5 and 9 he received intravenously 20,000,000 each of a polyvalent mixture of several strains of B. typhosus in addition to his own organisms.

The provocative injections produced a very slight hyperleukocytosis, blood cultures taken immediately after the clinical reaction remained sterile. Stool and urine cultures taken daily for 14 days failed to demonstrate organisms that could be identified with those isolated from the blood. The patient left the hospital on April 9 and returned to his work perfectly well on the 20th. On April 21 and 27 he received subcutaneously one billion each of the heatkilled, tricresolized bacteria, that is, the organism isolated from his blood. The agglutinations before and after the injections were as follows:

AGGLUTINATIONS BEFORE AND AFTER INJECTIONS OF BACTERIA

Date	Bacillus Isolated from Blood, Living	B. typhosus Formalinized	B. paratyphosus A Formalinized	B. paratyphosus B Formalinized
April 5	0	1:20 ++	0	0
April 8	0	1:20 ++	0	0
April 21	1:10 ++	1:20 +++, 1:40 ++	0	0
April 27	1:80 ++	1:40 +++	0	0
May 2	1:80 ++	1:40 +++	0	0

As several independent workers, by the use of the ordinary procedures, classified the bacillus isolated from the blood stream of the caretaker as a typhoid bacillus, the malady was also clinically diagnosed as a mild abortive form of typhoid fever in an aged and vaccinated man. Subsequent observations on the original milk and carbohydrate tubes and repeated agglutination tests threw doubt on the original identification and a more detailed study was contemplated as soon as other duties in the laboratory permitted. Such an inquiry became a necessity when another laboratory worker contracted an infection which was clinically diagnosed as typhoid fever and an organism similar to the one found in case 1 was isolated from the urine. The history of the second case is:

CASE 2.—A woman, aged 26, graduate student in bacteriology in this laboratory, complained of headache, general malaise, abdominal pains and remained absent from her work on June 2, 1919. For the last two months she had assisted in making thousands of agglutination tests of suspected typhoid colonies; in particular she had made several agglutination and fermentation tests with the bacillus isolated from case 1. In the course of these tests she examined also various plates that contained organisms of the B. enteritidis and B. paratyphosus B. rodent group. Her technic was clean and careful, and she always disinfected her hands thoroughly before leaving for meals. In July, 1918, she was vaccinated with T. A. B. Navy vaccine at Mare Island; each injection produced a moderately severe reaction. In October, 1919, she had a severe attack of pandemic influenza.

From June 2 until June 15, neither of us saw the patient, who lived out of the city, but a tentative diagnosis of abortive typhoid was suspected by the physician. To confirm this diagnosis we offered our services and con-

ducted repeatedly laboratory tests which are summarized in table 1. About June 20, the patient having been afebrile was permitted to leave the bed. She suffered a relapse on June 22 and was after that attended regularly by a physician and a nurse. The temperature chart available shows two typical enteric fever relapse curves, one extending from June 28 to July 12 and the other from July 14 to July 25. The only clinical data available state that the course was severe, accompanied in the last relapse by delirium. No rose spots were noticed, the spleen was never distinctly palpable but the pulse and blood count were characteristic for typhoid fever. Beginning June 28 she received a high calory carbohydrate diet. Her recovery was uneventful and complete. Our laboratory findings, which have a bearing on the problem to be discussed, are for the sake of clearness presented in tabulated form.

TABLE 1

Laboratory Findings in Case 2

Date	Leukocytes	Agglutination		Blood Cultures	Urine Cultures
		B. Typhosus	B. Para A, B, Dysenteriae, B. Melitensis		
June 15	7,300	1:20	0	Sterile	Negative
June 25	9,300	1:100	0	—	Negative
July 4	—	1:640	0	—	Positive, 6 colonies
Aug. 27	—	1:1000+	1:10–1:100	—	—

The facts stated, in conjunction with a consideration of the bacteriologic findings, are of considerable interest. A laboratory worker vaccinated against typhoid and paratyphoid developed a clinically typical typhoid fever infection. Agglutination and blood cultures failed to support this diagnosis until a bacillus identical in every respect to the one found in case 1 was isolated from the urine in the fifth week of the disease. Blood cultures were taken only in the second week, when the temperature was declining and the stool cultures were undoubtedly negative on account of the high calory carbohydrate diet, which had been given to the patient since June 10. The patient apparently was shedding organisms in the first two to three weeks' period of her illness as was indirectly demonstrated by the occurrence of another typical case of typhoid fever in her household. The history of this patient is:

Case 3.—The father of patient in case 2, aged 70, unvaccinated, complained of malaise and headache on July 24. From that date until August 9 his temperature rose to 102 and 104 F., but he was not sufficiently ill to follow the advice of his physician which was to remain in bed. On August 9 agglutination was found positive by a board of health laboratory, and he was subsequently kept in bed. From the few data available it is evident that his typhoid infection was typical, very severe and ended fatally on Sept. 8, 1919. The clinical diagnosis was well supported by a few examinations we were able to conduct on the patient on Aug. 25, 26, and 29, 1919.

FINDINGS IN CASE 3

Date	Agglutination		Stool Culture	Urine Culture
	B. typhosus Formalinized	Case 1 Bacillus, Living		
Aug. 25	....	....	∞ typical B. typhosus	
Aug. 26	....	....	....	∞ B. typhosus
Aug. 27	....	....	....	300 million B. typhosus per c c of urine 4 hours after collection
Aug. 29	1:400 +++; 1:800 ++	1:600 +++; 1:800 ++	Almost pure B. typhosus	90 million B. typhosus per c c of urine 9 hours after collection

As Sch. never entered the room of the patient and the most scrupulous precautions in sterilizing all secretions had been taken by the nurse who attended case 2 since June 28, it was for a considerable period impossible to connect his infection with the one of his daughter, discussed in case 2. Sch. had remained in his home and our most searching epidemiologic inquiries failed to find an outside source where he could have contracted the disease. On further detailed analysis of the circumstances leading to the illness of her father, the daughter remembered that on or about June 20 when very ill she prepared unknown to her mother who attended her a specimen of her own stool and instead of sterilizing the applicator, threw it in the toilet. On July 12 a plumber was called to clean the clogged siphon. Sch. assisted him, removed and handled the applicator and commented to his wife and the nurse on the negligence of the person, who threw the piece of wood in the lavatory. Twelve days later on July 24 he noticed the initial symptoms of his typhoid infection.

The occurrence of this indirect contact case would in itself, as from an epidemiologic point of view, be of little value, but in correlation with the bacteriologic findings, the history can be appreciated and analyzed more carefully.

BACTERIOLOGIC IDENTIFICATION OF THE ORGANISMS ISOLATED FROM CASES 1, 2 AND 3

As stated in the histories, the bacillus isolated from the blood stream of case 1 behaved irregularly when tested by more detailed carbohydrate and serologic tests; the organism found in one urine sample of case 2 corresponded with the organism of case 1, with the exception of a marked hyperagglutinability in the first 16 transplants on digest or veal agar. The organism isolated from case 3 was easily identified as a typical typhoid bacillus. In the course of the epidemiologic analyses of the recorded findings it became necessary to compare the isolated organisms with the various typhoid strains to which the laboratory workers were suspected of having previously been exposed. It was, however, impossible to demonstrate conclusively the strain or strains of B. typhosus, which by passing through the body of case 1 had

become altered to an irregular typhoid bacillus, nor did we collect observations which could prove the infections were the result of an irregular B. enteritidis. Before discussing the various tests employed for the identification we state briefly the technical procedures used.

Blood cultures were made with from 5 to 10 c c of blood in glucose veal infusion broth (P_H 7.0) and peptone-glycerol-ox bile. Stool and urine specimens were plated on brilliant green-eosin-peptic digest agar. Urine samples were also enriched with an equal amount of peptic digest broth.

The isolated colonies of gram-negative nonlactose fermenting organisms were purified by repeated successive plating on peptic digest or veal agar. The three strains studied in this paper were also isolated by Burri's method as one cell cultures. The progenies of three cells of each strain were studied in peptone water-potassium phosphate-sodium chlorid-carbohydrate solutions with Andrade's or China blue rosolic acid indicator, Witte's peptone solution, bromcresol purple milk, neutral red, orcein and malachite green solution in 0.5% meat extract agar, and rhamnose-veal agar. The P_H^+ reaction of all mediums used was adjusted to 7.0-7.2. It will be shown in another paper that strain "I 75" of case 1 when first isolated was alkaline tolerant; the growth curve showed a marked plateau extending from P_H^+ 6.8 to 7.8. All tests reported have been repeated at least three times and the findings with a few minor exceptions to be discussed in detail remained constant. The parasitic strains differed in no way biochemically from the saprophytic ones; however, this statement cannot be applied to some of the serologic findings on the saprophytized offsprings of the two strains "I 75" and "Chr. 76." It appears advisable for the sake of clearness to discuss the various characteristics under separate headings.

Morphology.—The three strains "I 75," "Chr. 76" and No. 49 are morphologically indistinguishable from the typhoid type strain "Rawlings"; they are gram-negative and actively motile. They show differences in size during their growth on mediums identical with those described by Clark and Ruehl;[7] on very alkaline mediums filamentous rods are frequently noted.

Surface Colonies.—On dye mediums or on plain agar the parasitic strain of "I 75" and "Chr. 76" produced vine leaf shaped granular colonies. As a rule the colonies were always somewhat larger and the characteristic growth permitted recognition of the irregular strains in a mixture with a typical B. typhosus. The inside structure shows a rather fine striated network of furrows, which are readily visible with the naked eye. Indeed the colonies correspond in many respects with those recently described by v. Lingelsheim and Sachs-Müke[8] as so-called Q-strains. Recent tests with the more saprophytic strains produced irregular grayish or slightly yellowish lobulated colonies, which developed raised centers and some isolated colonies may occasionally show indications of slimy edges. These changes occur only when the plates after incubation for 18 hours at 37 C. are kept at room temperature and again the mucous appearance of the edges is only slight in comparison with those constantly noted on typical paratyphoid B. strains. On gelatin plates the typical leaf-like appearance of the colonies is more pronounced than on the agar plates, the medium is never liquefied.

[7] Jour. Bacteriol., 1919, 4, p. 615.

[8] Centralbl. f. Bakteriol., 1913, 68, pp. 577 and 582.

Lead Acetate Reaction.—The medium prepared according to Jordan and Victorson[9] is slowly reduced without the production of gas; the hydrogensulfide reaction is identical with the one noted for typical typhoid strains tested simultaneously.

Carbohydrate Reactions.—Strain "I 75" and "Chr. 76" ferment without gas production the following carbohydrates: glucose, levulose, galactose, mannose, mannite, maltose, xylose, dextrin, arabinose (3 times of 5 tested), dulcite and rhamnose. Strain "49" failed to ferment arabinose, dulcite and rhamnose in the observation period of 30 days. It is generally stated in textbooks and emphasized by Winslow, Kligler and Rothberg[10] in their studies on the classification of the colon typhoid group, that the type strain "Rawlings" does not attack arabinose, dulcite or rhamnose. Recent studies by Teague and Morishima[11] confirming previous observations made by Penfold,[12] Wagner,[3] Ditthorn[13] and others indicate that at least 6% of their typhoid cultures showed acid production in arabinose and from 14 to 37% in dulcite broth, when the period of observation was extended to 30 days. Of the 14 typical typhoid strains, which are under suspicion of containing the strain responsible for the infection in case 1 and used by us for comparison, 2 or 14% fermented repeatedly arabinose on the 7th or 14th day and 6 or 48% acted on dulcite in the one test, in which all strains were tested simultaneously. Repeated tests of our strain "I 75" and "Chr. 76" in arabinose-peptone-indicator solution confirmed the observations of Teague and Morishima that the acid production in this carbohydrate is irregular. In an early series with the parasitic strains acid production was noted in from 4 to 6 days; in another series with the saprophytic strains the reaction was delayed for 15 and even 24 days. On the other hand, the fermentation of dulcite was fairly regular; as a rule, acid was formed in from 2 to 4 days; in one series of tests a delay of 8 days was recorded. In some tests with dulcite the indicator was slightly reduced.

On endoplates prepared with arabinose instead of lactose, strain "I 75" and "Chr. 76" produced inside of the large isolated colonies in from 5-7 days one or several bud-like daughter colonies. Transplants from the papillae fermented arabinose in 24 hours. These observations on fuchsin-arabinose-agar are in many respects similar to those described by one of us (K. F. M.) for the paracolon bacilli isolated from calfscour's.[14]

On dulcite-endoplates also red papillae are produced about the 8th to 10th day, but transplants from these behaved irregularly; an observation which we found confirmed by the recent publication of Teague and Morishima.

The fermentation of rhamnose or isodulcite is regularly noted in any liquid medium chosen. Sometimes 3-5 days elapse before distinct acid reaction is shown by the indicator; in some series the acid production was confined to the flocculent growth sediment of the tubes and only 3-4 days later the acidity diffused throughout the liquid. According to Krumwiede, Kohn and Valentine[15] and Winslow, Kligler and Rothberg,[10] who have recently tested a series of typhoid strains, it is generally believed that the B. typhosus does not ferment this particular carbohydrate and the bacillus can therefore readily be differ-

[9] Jour. Infect. Dis., 1917, 21, p. 554.
[10] Jour. Bacteriol., 1919, 4, p. 472.
[11] Jour. Infect. Dis., 1920, 26, p. 52.
[12] Jour. Hyg., 1912, 12, p. 195.
[13] Centralbl. f. Bakteriol., 1912-13, 67, p. 497.
[14] Jour. Infect. Dis., 1916, 19, p. 700.
[15] Jour. Med. Research, 1918, 38, p. 89.

entiated from the members of the paratyphoid group. Penfold,[16] on the other hand, states that "growth of B. typhosus on isodulcite broth frequently does not produce acidity though it may do so as early as one week." Thus it is quite evident that this pentose in a liquid substratum is not of much value for distinguishing irregular strains of B. typhosus from nongas-producing paratyphoid strains.

Rhamnose-Agar Papillae Reaction.—In this connection it should be recalled that R. Müller[17] and later Penfold,[16] Saisawa,[18] Wagner,[3] Teague and Morishima[11] consider the development of daughter colonies on rhamnose agar a specific reaction for typhoid bacilli. In a series of tests with a small amount of rhamnose available we were able to confirm this specificity. Strain 49 and 4 other typhoid strains, representatives of the three groups of Hooker's serologic classification produced immunerable dense papillae in from 48 to 72 hours. Strain "I 75" and "Chr. 76" in spite of vigorous growth developed only small daughter colonies about the 10th or 12th day of incubation; they rarely reached the size of those noted with the typical typhoid strains and always remained translucent. Strain "I 75" and "Chr. 76" evidently differ in the rhamnose-papillae reaction from the typical typhoids which in part explains their ability to ferment this carbohydrate by acid production. The freshly isolated, as well as the saprophytic, strains behave in an identical manner. Thus far no reversion to the true type has been observed.

Raffinose-Agar Papillae Reaction.—Neither of the three strains concerned in this publication produced papillae on raffinose agar. The animal strains of B. paratyphosus B and B. enteritidis to which the workers had been exposed, all showed centrally located daughter colonies.

Little need be said with regard to the fermentation of xylose. We fully agree with Teague and Morishima that the so-called xylose nonfermenters are in reality slow fermenters. Strain "I 75" and strain "Chr. 76" were rapid xylose fermenters and maintained this property when repeatedly tested during the last 8 months.

Bromcresol-Purple Milk.—Both strains "I 75" and "Chr. 76" are characterized by rapid alkaline production in milk. The initial slight acidity is changed on the 3rd to the 5th day to a decided alkalinity which progressively increases and leads to saponification of the milk fat on the 15th to 30th day. The rapidity with which the reaction changes from a P_H^+ 6.6 to P_H^+ 9.0 in this medium has somewhat slowed down in these cultures kept for 6 months on plain peptic digest agar. The two strains as second and third generations isolated from the human body produced a deep purple reaction (P_H^+ 8.6) inside of 5 days. Quite recently tested, at least 10 to 15 days elapsed until the same degree of alkalinity under the same conditions was noted. We have made similar observations on several strains of B. sanguinarum kept on agar for nearly 4 years; originally rapid they have gradually changed to slow alkali producers. It was noted in a series of tests with strain 49 that often at the end of 30 days' incubation about 50% of the inoculated milk tubes gave decided alkaline reactions. For example, transplants made from 12 isolated colonies on plain agar into milk tubes of the same lot, produced after varying intervals the following reactions:

[16] Brit. Med. Jour., 1910, 2, p. 1672.
[17] Centralbl. f. Bakteriol., 1911, 58, p. 97 and Münch. Med. Wchnschr., 1909, 49, p. 885.
[18] Ztschr. f. Hyg. u. Infektionskrankh., 1913, 74, p. 61.

REACTIONS OF TRANSPLANTS FROM TWELVE ISOLATED COLONIES ON PLAIN AGAR INTO MILK TUBES OF SAME LOT

	Milk Reaction		
	On 1st Day	On 16th Day	On 30th Day
1. Smooth colony	Pн.6.8	Pн+.6.2	6.2
2. Smooth colony	Pн.6.8	Pн+.6.2	6.2
3. Smooth colony	Pн.6.8	Pн+.7.7	7.8
4. Smooth colony	Pн.6.8	P+н.7.7	7.7
5. Smooth colony	Pн.6.8	Pн+.7.7	8.0
6. Smooth colony	Pн.6.8	Pн+.6.2	6.2
7. Smooth colony	Pн.6.8	Pн+.7.7	8.5
8. Smooth colony	Pн.6.8	Pн+.7.3	7.3
9. Vine leaf like colony	Pн.6.8	Pн+.6.8	6.8
10. Vine leaf like colony	Pн.6.8	Pн+.6.8	7.0
11. Lobulated colony	Pн.6.8	Pн+.7.5	7.7
12. Lobulated colony	Pн.6.8	Pн+.6.2	6.2
Strain "I 75." One vine leaf colony	Pн.6.8	Pн+.8.5	9.0 gelatinized

The differences in the final P_H^+ reaction are merely the result of differences in the rate of multiplication of the selected colonies. Strain "I 75" in comparison with strain 49 possesses a more rapid growth rate, which in turn gives rise to alkaline split products in a correspondingly shorter time interval. It is as yet undecided, whether the alkaline reactions were caused primarily by the oxidation of the salts of citric acid to alkaline carbonates as recently suggested by Ayers, Rupp and Johnson[19] or the result of a true alkali fermentation. Some incomplete data at our disposal support strongly the contention of the workers mentioned.

Bradley,[20] Krumwiede, Pratt and Kohn[21] and recently Jordan[22] pointed out that the differences in the milk reaction of the various paratyphoid strains are probably due merely to a difference in the rate of multiplication. A similar mechanism seems operative in the milk reactions of the typical and the so-called "blue typhoids." Even different members of one and the same strain may develop in milk rapid or slow alkali-producing offsprings. Bromcresol purple milk can therefore not be recommended as a suitable differential medium for the classification of irregular strains.

Indol Production, Methyl Red Test and Hemolysis.—In one tube of Witte's peptone solution inoculated with the second generation of strain "I 75" a slight but definite indol reaction was noted with Ehrlich's reagent on the sixth day. Subsequent tests, including strain "Chr. 76," gave negative results. Through the observations of Andrejew[23] and Bull and Pritchett[24] it is known that irregular typhoid strains occasionally produce traces or even large amounts of indol. The methyl red test was positive. All 3 strains failed to produce hemolysis on blood-agar plates.

Reduction of Dyes.—Neutral red in 0.5% agar with or without glucose was not reduced by the 3 strains under discussion. A slight slow reduction (5 days) of malachite green and orcein agar was observed, but also constantly noted with the control typhoid strains.

[19] U. S. Depart. Agr. Bull., 782, 1919.
[20] Jour. and Proc. Roy. Soc. N. S. Wales, 1912, 46, p. 74.
[21] Jour. Med. Research, 1916, 35, p. 55.
[22] Jour. Infect. Dis., 1918, 22, p. 511.
[23] Arb. K. Gsndttsamte, 1910, 33, p. 363.
[24] Jour. Exper. Med., 1916, 24, p. 39.

The detailed findings may be briefly summarized: Biochemically, strain "I 75" and "Chr 76" differ from the true typhoid strains, including strain No. 49, by their intensified carbohydrate reactions; dulcite, rhamnose and arabinose broth in the order stated are acidified in a shorter time interval than is customarily recorded for B. typhosus. This accelerated ferment action may also be in part responsible for the rapid alkali production in milk, which we found apparently was the result of a more rapid and more intense growth in this medium than is ordinarily observed with typical typhoid strains. Both strains fail to produce papillae on raffinose agar, but do so on rhamnose plates.

Serologic Identification

A suspension of the gram-negative, motile bacilli, which grew in the blood culture of case 1, was promptly clumped by a polyvalent typhoid immune serum in a slide and in a macroscopic agglutination test. Additional determinations made with specific rabbit antiserums and suspensions of living bacteria of strain "I 75" placed this organism serologically with the typhoid bacillus. The reactions were always well marked with typhoid serum and coreactions with other serums were slight or absent. In the course of several months, when an attempt was made to determine more closely the group relationship of strain "I 75" it was found that this organism when preserved in a 0.1% formalinized salt solution was inagglutinable by B. typhosus serum, but had apparently acquired the property of being specifically clumped by B. enteritidis serums in maximum dilutions. This striking specificity of the killed in contrast to the living suspensions has been occasionally noted with other strains, but never to such a degree as was constantly encountered in the tests with strain "I 75." In the literature we found only the statements by Kafka,[25] Klemens,[26] Minelli[27] and others, that formalinized suspensions may show complete absence or marked reduction of coagglutination reactions. On the other hand, most writers agree that agglutination reactions with living bacteria must be considered more sensitive than those conducted with dead cultures. It is, however, evident that little attention has been paid to this phenomenon and a careful study of immunologic and physicochemical factors responsible for this differences suggest themselves. Repeated tests conducted during the last 8 months with at least 20 different formalinized and living suspensions always gave identical and uniform results, which are shown in table 3.

Strain "Chr. 76" was hyperagglutinable when first isolated and could only be tested after 30 successive transplantations on neutral peptic digest agar. By changing the electrolyte contents of the suspensions and serum dilutions, in using a 0.25% salt solution according to the method of Verzàr[28] specific reactions and slight coreactions occurred with typhoid serum and living suspensions. These preliminary tests placed, in our opinion, strain "Chr. 76" with the typhoid bacillus. Several months after the date of isolation when absorption tests were in the process of preparation agglutination tests with formalin-

[25] Centralbl. f. Bakteriol., 1906, 40, p. 247.
[26] Berl. klin. Wchnschr., 1905, 42, p. 1269.
[27] Centralbl. f. Bakteriol., 1906, 41, p. 583.
[28] Centralbl. f. Bakteriol., 1917, 80, p. 161.

ized suspensions were undertaken. Again the same phenomenon, as already described for strain "I 75," became apparent, namely strain "Chr. 76" was inagglutinable in formalinized killed suspensions; it was, however, specifically

TABLE 3

AGGLUTINATION REACTIONS

Antiserums	Antigen "I 75"		Antigen "Chr. 76"		Antigen "49"	
	Living Second Generation	Formalinized 0.1% 21st Generation	(Living Second Hyper-agglutinable) 15th Generation in 0.25% Saline Specific Agglution	Formalinized 0.1% 30th Generation	Living Second Generation	Formalinized 0.1%
Polyvalent B. typhosus (1:40,000)	1:600 (blood broth) (bile broth)	0	1:1000+++	0	1:20,000	1:40,000
Hooker's Group I: B. typhosus "9" (1:10,000)	1:2000 (20th gener.)	0	1:1000+++	0	1:10,000 (10th gener.)	1:10,000
Hooker's Group I: B. typhosus "11" (1:10,000)	1:200+++ (20th gener.)	0	....	....	1:10,000 (10th gener.)	>1:10,000
Hooker's Group II: B. typhosus "Rawlings" (1:8,000)	1:600+++ (20th gener.)	0	1:600++	0	1:8,000 (10th gener.)	1:6,000
Hooker's Group II: B. typhosus "Dorset" (1:20,000)	1:400+++ (20th gener.)	0	....	....	1:20.000 (10th gener.)	1:20,000
Hooker's Group III: B. typhosus "Hopkins" (1:10,000+++)	1:600+++ (20th gener.)	0	1:600+++	0	1:10,000 (10th gener.)	>1:10,000
Hooker's Group III: B. typhosus "1" (1:6.000+++)	1:200 (20th gener.)	0	1:400+++	0	1:6,000 (10th gener.)	1:8,000
B. paratyphosus B polyvalent (1:20,000)	1:20+++	0	1:80++	0	<1:200	0
B. paratyphosus B human (1:10,000)	....	0	....	....	<1:100	0
B. paratyphosus B avian (1:20,000)	....	1:50±	....	....	....	0
B. paratyphosus B rodent (1:20,000)	....	0	....	....	....	0
B. paratyphosus A (1:40,000)	1:40++; 1:60+	0	1:800+++	0	<1:100	0
B. sanguinarium (1:1,000)	1:200	0	1:100++	0	1:200	1:100
B. pullorum (1:2.000)	1:100	0				
B. enteritidis human III (1:20.000)	....	1:2,000	....	1:2,000	<1:100	0
B. enteritidis rodent I (1:10,000)	<1:50	1:2,000	....	1:2,000	....	0
B. enteritidis calf. (1:10,000)	....	1:2,000	....	1:2,000	....	0
Normal rabbit serum	1:10	0	1:120	0	<1:10	0

agglutinated by B. enteritidis-serums. Strain "Chr. 76" in living suspensions is somewhat more readily sedimented and clumped by typhoid and paratyphoid serums than strain "I 75," but from the standpoint of the serologic data presented in table 4 the two strains must be considered as identical. The parasitic strains differ from the saprophytic ones by their ability of being readily agglutinated by B. enteritidis serums. Strain No. 49 behaves serologically like a

typhoid bacillus. No changes in agglutinability have been noted during the last 4 months. Standardized suspensions are specifically agglutinated by typhoid serums and group reactions are only noted with B. sanguinarum-serums.

The fact that formalinized killed suspensions of strain "I 75" and "Chr. 76" were not agglutinated by typhoid serums made the original diagnosis rather questionable and it was thought possible to determine the exact position of the bacteria under consideration by the use of a specific serum prepared with strain "I 75." On account of the high toxicity of this strain we succeeded only after many attempts in producing a highly specific and potent serum of a titer of 1 : 200,000.

TABLE 4

TESTS WITH STRAIN "I 75" ANTISERUM

Antiserum for Strain "I 75"		Living Suspended 0.1% Formalinized Salt Solution	Killed in 0.1% Formalinized Salt Solution
	Strain "I 75"	1:200,000	1:20,000
	Strain "Chr. 76"	1:100,000	1:80.000
	Strain "Sch. 49"	1:1,000+++; 1:2,000++	0
	B. typhosus "Rusk"	1:4.000+++	0
	B. typhosus "Blair"	1:10,000+++	—
	B. typhosus "Singleton"	1:6.000+++	0
	B. typhosus "Jacobs"	1:200+++	—
	B. typhosus "Blunt"	1:1,000+++	—
	B. typhosus "Moffitt"	1:200+++	—
	B. typhosus "Kleeberg"	1:6,000+++	—
	B. typhosus "Houston"	1:1,000+++	—
	B. typhosus "Cordona"	1:8,000+++	—
	B. typhosus "15"	1:4,000+++	—
	B. typhosus "Kearney"	1:4,000+++	0
Hooker's Group I	B. typhosus "52"	1:200++	0
Hooker's Group I	B. typhosus "40"	1:2,000+++	0
Hooker's Group I	B. typhosus "11"	1:200	0
Hooker's Group II	B. typhosus "Dorset"	1:2,000+++; 1:4.000++	0
Hooker's Group II	B. typhosus "Rawlings"	1:6,000+++; 1:8,000++	0
Hooker's Group III	B. typhosus "1"	1:2,000+++; 1:10,000++	0
Hooker's Group III	B. typhosus "3"	1:4,000+++; 1:6,000+	0
B. paratyphosus "Human 26"		1:200+++	0
B. paratyphosus A "13, 15, 16"		1:400+++ 1:1,000+	0
B. sanguinarium "5"		1:100+++ 1:4,000++	0
B. enteritidis, strain 1, origin "rat"		1:200,000	1:20,000+++
B. enteritidis, strain 2, origin "A. M. N. H., unknown"		1:200,000	1:20,000+++
B. enteritidis, strain 3, origin "Strassburg, human"		1:200,000	1:10,000+++
B. enteritidis, strain 6		>1:200,000	1:40,000+++
B. enteritidis, strain 13, origin "Calfscours"		1:200,000	1:20,000+++

It is clearly indicated that such a serum gave with living suspensions of a variety of typhoid and also paratyphoid A bacilli, pronounced and fairly uniform coreactions. On the other hand, the "I 75" antiserum agglutinated in formalinized suspensions only its own organism, strain "Chr. 76" and a number of B. enteritidis strains isolated from various sources. The coreactions obtained with the 11 typhoid strains, which are under suspicion of having been the sources for the infection of case 1, and the creation of strain "I 75"

were not sufficiently striking to stigmatize any particular one as being antigenically closely related to strain "I 75," and again, the strains which represent the 3 groups of Hooker's classification[29] are not influenced serologically by the "I 75" immune serum in such degrees that a relationship of our strain "I 75" to either one of these groups could be arbitrarily deducted. To be sure, the reactions appear more as group reactions which apparently embrace the entire typhoid-paratyphoid group.

At this stage of the serologic identification it was considered necessary to apply absorption tests, naturally using living suspensions as antigens, and absorbing the immune serum completely of their agglutinin content. Extensive experimental series with the organisms of the B. paratyphosus and B. melitensis groups have convinced us that only the complete removal of all immune substances will give comparable results. With highly potent serums, such as the one prepared with "I 75," the procedure of removal is very tedious; 4 to 8 saturations with living organisms are sometimes necessary to deprive the serum of its entire agglutinin content for the absorbing antigen. The technic used by us is similar to the one described by Taylor, a detailed account therefore appears superflous.

TABLE 5

Antiserums Agglutinate with Living Antigens After Complete Absorption

Strains	"I 75" with "I 75"	"I 75" with B. typhosus "9"	"I 75" with "Rawlings"	"I 75" with B. typhosus "3"	B. typhosus "Rawlings" with "I 75"	B. typhosus "3" with "I 75"	B. typhosus "Rawlings" with B. typhosus "3"
I "75"	0	1:200,000	1:200,000	1:200,000	0	0	0
Chr. "76"	0	1:100.000	1:100,000	1:100,000	0	1:40±	0
No. 49	0	1:40±	0	1:320	1:10,000	>1:5,000	1:40
Hooker's Group I							
B. typhosus "9"	0	0	0	1:40+	1:4,000	1:2,000	1:80
B. typhosus "11"	0	0	0	1:80	1:4,000	1:600	1:200
Hooker's Group II							
B. typhosus "Rawlings"	0	1:40±	0	1:320	1:10,000	1:2,000	1:600
B. typhosus "Dorset"	0	1:320	0	1:160	1:2,000	1:2,000	1:40
Hooker's Group III							
B. typhosus "Hopkins"	0	1:160	1:40	1:320	1:4,000	1:4,000	1:80±
B. typhosus "1"	0	1:320	1:640	1:640	>1:2,000	>1:5,000	0
B. typhosus "3"	0	....	1:320	0	1:160	1:2,000	0

0 indicates agglutination less than 1:40.

Strain "I 75" removes from its own immune serum all coagglutinins for the B. typhosus. On the other hand, a typical B. typhosus recently isolated and belonging to group I of Hooker's classifications removes from the serum of strain "I 75" the immune substances for his own strain and closely allied representatives of group II and III. The agglutinins for strain "I 75" remain quantitatively intact. A similar phenomenon takes place when this serum is absorbed with representatives of groups II and III. It is, however, apparent that a B. typhosus strain belonging to group III deprives the immune serum "I 75" in repeated tests incompletely of its agglutinins for the representatives of groups I, II and III. From the studies of Hooker the rather heterogeneous composition of this group is known and irregular reactions actually characterize this subgroup of typhoid bacilli. In a typhoid immune serum prepared with

[29] Jour. Immunol., 1917, 2, p. 1.

the type strain "Rawlings" group II the strain "I 75" removes its own group agglutinins, but the major typhoid agglutinin remains practically unaltered. The effect of "I 75" on an immune serum prepared with an organism of group III B. typhosus 3 is identical, and again, a B. typhosus of group III removes from a group II serum not only the agglutinins for his own group, but also those of strain "I 75" and group I simultaneously, thereby reducing the active substances for group II. These preliminary absorption tests will be enhanced along various other, particularly quantitative, lines as suggested in the recent publication by Andrewes and Inman,[30] but they are, so far as it concerns typhoid serums, sufficiently definite to draw certain deductions. Speaking in terms of agglutinin content of these serums, it is evident that a strain "I 75" serum contains, aside from its own major agglutinin, coagglutinins for groups I, II and III of Hooker's classification and absorption with representatives of these groups removes only these group-agglutinins. A "Rawlings" or a group III serum, on the other hand, has group agglutinins for "I 75" which can be specifically absorbed by this strain. Agglutinins for group III in a group II serum remove also the immune substances for strain "I 75."

Serologically strain "I 75" and "Chr. 76" belong to the typhoid group of bacteria; they differ antigenically from the three groups of Hooker; but are closely related to his heterogeneous group III. We are unfortunately not in possession of the typhoid strains used by Weiss[31] and therefore cannot state in which antigenic subgroup mentioned in his study strain "I 75" and "Chr. 76" should be placed. One point is certain: Our strains stand apart as a definitely differentiable type, even when using living cultures. Moreover, their relation to the typhoid group was shown only by the use of living suspension; formalinized antigens were either inagglutinable or highly specific.

Attention has already been called to the interesting fact that strain "I 75" and "Chr. 76" gradually acquired the ability to be agglutinated in living, and in killed suspensions as well, by B. enteritidis serums and vice versa. This group of organisms was uniformly agglutinated to the titer limit by the specific "I 75" immune serums. On the other hand, strain 49 repeatedly tested was not agglutinated by any of the available B. enteritidis serum. At first the observation was explained by the well-known fact that B. enteritidis serums and vice versa B. typhosus serums in many instances give striking coreactions. Already Durham[32] and later Kutscher and Meinecke,[33] Liefmann[34] and others called attention to this peculiar serologic relationship of certain B. enteritidis strains to B. typhosus. Absorption tests, however, separated the two organisms in a decisive manner. Thus it would appear to be a simple procedure to determine whether our strains are true B. typhosus or true B. enteritidis. Our absorptions test produced, however, paradoxical results (see table 3).

Complete removal of the B. enteritidis agglutinin in a "I 75" immune serum deprives this serum also of the same substances for strain "I 75" and again a B. enteritidis serum absorbed with the irregular strain "I 75" or "Chr. 76" fails to agglutinate all of the B. enteritidis strains tested. Judging from these paradoxical results, which were repeated with various other completely and incompletely absorbed serums, we should conclude that strain "I 75" and "Chr. 76" are typical nongas-producing B. enteritidis strains. We searched in vain for similar observations in the literature, but could only find the references

[30] Medical Research Committee, Special Report, Series, No. 42, 1919.

[31] Jour. Med. Research, 1917, 31, p. 135.

[32] Lancet, 1898, 1, p. 154 and Brit. Med. Jour., 1898, 2, p. 588.

[33] Ztschr. f. Hyg. u. Infektionskrankh., 1906, 52, p. 30.

[34] München med. Wchnschr., 1908, 55, p. 159.

already mentioned. Christiansen,[35] the only writer who is thoroughly familiar with the nongas-producing B. enteritidis or paracolon strains, failed to conduct absorption tests probably because his serum coagglutinated typhoid bacilli in dilutions, which did not suggest such procedures. Until extensive studies with a large number of B. typhosus and B. enteritidis strains have demonstrated the antigenic relationship of these bacteria, we only record our observations and abstain for the present from offering an explanation. It is not unlikely that the B. enteritidis coreaction is characteristic for atypical typhoid strains and in this respect may have considerable diagnostic value and may even strengthen our conception of the typhoid nature of strain "I 75" and "Chr. 76."

In this connection attention is directed to the observations of Sobernheim and Seligmann,[36] which indicate a peculiarly marked lability of the antigenic properties of many B. enteritidis strains. Two old laboratory strains of this organism showed a transformation of their biologic properties, which was frequently combined with changes in the cultural characteristic. Careful plating methods demonstrated a number of daughter colonies, which apparently represented the transitional stages between the original and the finally transformed irregular strains. These observations are suggestive when we recall that our atypical strains acquired agglutinability for B. enteritidis serums in the course of a saprophytic life on agar slants. Neither the biochemical functions of our strains, nor the susceptibility for specific agglutination with typhoid serums have, however, changed in the course of at least 150 transplants. This and similar observations have convinced us that all future publications on pathogenic micro-organisms should definitely state whether the biologic and biochemical studies recorded were made on parasitic or saprophytic offsprings of the original culture.

Identification by Pathogenicity and Protection Experiments.—The freshly isolated strain "I 75" was exceedingly toxic for rabbits; the symptoms and anatomic findings differed in no respect from those commonly seen in animals intoxicated by true typhoid bacilli.

Guinea-pigs of 250-300 gm. of weight succumbed to intraperitoneal inoculations of from 60-100 million living organisms. Careful immunization with heat-killed organisms even by subcutaneous application of the inoculum is difficult; about 50% of the guinea-pigs show progressive emaciation without organic changes or lesions commonly noted in paratyphoid infections. Rats fed for one entire week with broth culture of "I 75" eliminated the fed bacteria, but remained clinically well.

Protection Experiments.—Recent studies conducted in this laboratory and to be published elsewhere demonstrated that the tissues of typhoid immune and nonimmunized rabbits destroy in a given time interval (24-48 hours) approximately the same number of intravenously inoculated typhoid bacilli. On the other hand, paratyphoid immune rabbits can apparently dispose of an infection produced by an intravenous inoculation of paratyphoid organism more rapidly and more completely than the nonimmune animals. This principle was applied to the identification of strain "I 75."

Exper. 1:—On Dec. 16 rabbit 1, which had been intensively immunized with dead and living B. typhosus "Rawlings" (agglutination titer of the serum 1: 6000), rabbit 2, immunized in an identical manner with the strain "I 75" (agglutination titer of the serum 1: 100,000) and a normal rabbit of the same litter and weight (agglutination titer $<$1:10) were inoculated intravenously with 1 c c each containing 6.400 million living orgnisms of strain "I 75." On

[35] Centralbl. f. Bakteriol., 1914, 74, p. 474.

[36] Deutsch. med. Wchnschr., 1910, 36, p. 351.

Dec. 17, seventy-four hours after the injection of the infective dose, rabbit 3 was profoundly intoxicated and showed rapid breathing and diarrhea. The two immune animals appeared less active and ate little. All three rabbits were exsanguinated under ether; the organs were removed aseptically and portions of the same were pulped with sand and saline in sterile mortars and diluted in such proportions, that each cubic centimeter of saline contained 100 mg. of tissue pulp. This material was plated as dilutions in peptic digest agar. The plates were counted after 48 hours' incubation at 37 C. Table 6 illustrates the average number of viable bacteria demonstrated in the tissues and in the blood stream.

TABLE 6

EXPERIMENT I: INTRAVENOUS INJECTION OF 6,400 MILLION ORGANISMS. SACRIFICED AND TISSUES PLATED 24 HOURS AFTER INJECTION

Tissues	Rabbit 1 Immune to B. typhosus "Rawlings"	Rabbit 2 Immune to Strain I 75	Rabbit 3 Normal
Agglutination titer	1:6,000+++	1:100,000+++	<1:10
	Per 100 mgm. of tissue. The following Grew After 24 Hours' Incubation	Per 100 mgm. of tissue. The following Grew After 24 Hours' Incubation	Per 100 mgm. of tissue. The following Grew After 24 Hours' Incubation
Liver, left and center lobe	12,000	20,000	480,000
Liver, right and center lobe	30,000	15,000	720,000
Bile	3 per 0.8 c c	0	238,000,000 per 1.7 c c
Gallbladder wall	90	0	18,000
Spleen	380,000	110,000	6,300,000
Bonemarrow	13,200	70,000	1,680,000
Mesenteric lymphnodes	180	400	12,400
Kidneys	72	6,700	34,000
Lungs	17,500	1,000	640,000
Heart blood	....	2,400	52,000
Carotis blood	200	....	48,000
Duodenum	Negative for B. I 75	1 colony of B. I 75	20 colonies of I 75
Ileum	150 colonies per loopful of intestinal content	2 colonies of B. I 75	

It is quite evident that the normal animal is less readily capable of destroying the intravenously inoculated bacteria of strain "I 75" than the immune one. The profound intoxication is indicated by a high bacterial count of the bone marrow, an observation which has recently been emphasized by J. T. Parker. The bile and spleen are also heavily infected. On the other hand, there is little difference between the animal immunized against the infecting strain and the one protected against the "Rawlings" organism. Both rabbits are destroying the inoculated organisms in the chosen time interval of 24 hours in approximately equal proportions. From our extensive experience with this particular method of immunity research already referred to we are justified in concluding that strain "I 75" behaves in the immune and normal rabbit like a paratyphoid organism, but that apparently no differences exist between the destructive forces of the animal immune to the infective strain "I 75" and the one which is only protected against the type typhoid strain "Rawlings." This experiment again supports the contention that strain "I 75" is antigenically closely related to the typhoid bacillus.

Bull and Pritchett[24] and recently J. T. Parker[37] have emphasized the fact that rabbits immunized with typhoid bacilli are highly and specifically resistant to intoxication with this organism. They withstand, as a rule, from 30 to 40 lethal doses of the living bacilli. Unfortunately no experimental data are available which prove conclusively that the toxic substances derived from organisms of the typhoid-paratyphoid group are strictly specific and our tests along these lines have not sufficiently matured to enable us to express a final

[37] Jour. Med. Research, 1919, 39, p. 301.

opinion. It is therefore with some hesitancy that we record some protection tests, which in themselves are very suggestive and which should encourage further inquiry along these lines.

Exper. 2:—One rabbit immune strain "I 75." 2 rabbits to different strains of typical B. typhosus, 1 rabbit to B. paratyphosus B, 1 to B. coli and 2 controls were injected with 50 lethal doses of strains "I 75." The animals succumbed after the following time intervals:

TIME IN WHICH ANIMALS SUCCUMBED TO LETHAL DOSES

	Died
Normal rabbit	3 hours, 50 minutes after the injection
Normal rabbit	4 hours, 10 minutes after the injection
Immune to B. paratyphosus	5 hours, 5 minutes after the injection
Immune to B. coli	5 hours, 25 minutes after the injection
Immune to B. typhosus polyvalent	23 hours after the injection
Immune to B. typhosus polyvalent	26 hours ofter the injection
Immune to Strain "I 75"	36 hours after the injection

It is a known fact, that normal rabbits vary considerably in their resistance to bacterial toxins of the typhoid group. This in part explains the unfortunate use of an intoxicating dose which also proved fatal in the specifically immune rabbit. The results could therefore be made more definite, but at least they indicate, as presented, that true typhoid bacilli protect to a certain degree against strain "I 75" and that paratyphoid and colon immune rabbits succumb to the intoxication as readily as the nonimmune ones.

We also attempted in a series of immune and normal guinea-pigs to determine the distribution and destruction of strain "I 75." Thus far we have noted exceedingly interesting paradoxical results, namely, the specifically and the typhoid immune guinea-pigs succumbed to the infection in contradistinction to the normal animals which remained alive. Until we have sufficiently often repeated these observations and can offer an explanation for this phenomenon we are withholding for the present the citation of a detailed experiment.

DISCUSSION

Before entering into a discussion of the various problems that suggest themselves in the analysis of the data, it is necessary to present a summary of the events as they appear in our interpretation.

An animal caretaker, who had intimate contact with secreta and cadavers of typhoid experimental animals, contracted an abortive attack of typhoidal fever. The short duration of his illness is probably due to his age immunity and to the prophylactic vaccination which had been administered to him, one and one half years previous to his infection. On only one occasion was an irregular typhoid strain isolated from his blood stream. His Widal reaction and stool and urine cultures were always negative. About two months later a vaccinated laboratory worker, who was experimenting with the irregular strain and also with the cultures of B. typhosus used for our experimental work on animals, developed a severe attack of typhoid fever. An irregular strain of B. typhosus identical with the strain isolated from

the caretaker was demonstrated on one occasion in her urine. Repeated blood, stool and urine culture during the relapse periods were negative. Her unvaccinated aged father contracted typhoid fever twelve days after handling a wooden spatulum, which had been used by the patient to prepare her stool specimen for shipment to laboratory. The stool and urine of this case contained typical typhoid bacilli. From the standpoint of the bacteriologist the variants of the B. typhosus may have originated in the following manner: The highly immune caretaker atavistically changed one or several typical strains of B. typhosus to the irregular strain described as "I 75," which in time caused a second severe infection in a vaccinated young woman. Her strain in passing through the tissues of an old, nonimmunized man reverted to a typical B. typhosus.

The observations recorded in the foregoing paragraphs deserve, however, a more detailed consideration from three different points of view, namely: (1) epidemiologic, (2) clinical and (3) bacteriologic.

1. *Epidemiology*.—As already outlined in the history of case 1, we feel convinced on epidemiologic grounds that our animal caretaker contracted his infection through intimate contact with heavily infected typhoid secreta of rabbits and guinea-pigs. Most painstaking inquiries which were met by a liberal cooperation on the part of our janitor failed to reveal any possibility of outside connections with acute, latent or carrier typhoid fever cases. His whereabouts the last four months previous to his transitory illness were readily traceable on account of the illness of his wife and his compulsory functions as a nurse. The only determinable source was the infection from our experimental animals. It is well known to workers in this field of experimental pathology that renal (in the first) and gallbladder carrier rabbits (in the second place), as a rule, may shed enormous numbers of living, virulent typhoid bacilli. The sawdust bed of such carrier cages regularly contains demonstrable B. typhosus, and again the handling of typhoid animal cadavers is connected with even greater danger of exposure. Even with the average amount of care it is unavoidable that such material soil the hands of the cleaner or cremator. For this reason it has been our policy to protect our personnel by vaccination, repeated in from one to two years. Kisskalt,[38] in his recent summary of typhoid laboratory infections, mentions several cases which resulted from contact with animal material, particularly rabbit typhoid carriers.

[38] Ztschr. f. Hyg. u. Infektionskrankh., 1915, 80, p. 145.

One case is cited in which a laboratory janitor contracted typhoid fever through eating his meals in a stable where a goat had been inoculated with living typhoid bacilli. Thus it is apparently not an uncommon occurrence that such experimental material serves as a potent source for enteric fever infections. It may, however, be stated that undoubtedly some as yet unknown factors must be concerned, because the writers have in the last four years repeatedly aspirated or otherwise come in contact with heavily infected animal material, and neither of the two has contracted typhoid fever. It is our belief that the prophylactic vaccination practiced every 12 months is mainly responsible for the fortunate outcome of the unavoidable accidents.

Epidemiologically, case 2 is again explained on circumstantial evidence only. The patient is personally convinced that she contracted the infection in the course of her laboratory work in making innumerable slide agglutination tests. It is difficult, however, to state the date on which she probably became infected and particularly in the light of the interesting bacteriologic findings, it would be invaluable to know whether she infected herself with strain "I 75" isolated from case 1, or with one of the many typhoid strains in use at the time theoretically accountable for her illness. One thing is certain, that the identical organisms were tested by her which are considered responsible for case 1. An infection outside of the laboratory has been completely ruled out by a searching study of a possible source. Her first clinical symptoms developed about 50 days after she had handled strain "I 75," according to her records. There are two possibilities which may help to explain the identity of the strain "Chr. 76" isolated from case 2 with strain "I 75," either in the vaccinated person strain "I 75" remained latent or one or several of the experimental strains of B. typhosus were in her tissue transformed in a similar manner into an atypical strain as we assume it to be case for strain "I 75." Personally, we believe that the worker handled the culture "I 75" perhaps unknown to herself in a period shortly before she developed the clinical signs of typhoid fever. Repeated attempts to prove experimentally or otherwise the suggested explanation have failed and the identity of culture "I 75" and "Chr. 76" is the only tangible link which establishes the connection of case 2 with case 1.

The practical epidemiologist has no difficulty in explaining case 3. The handling of a spatulum used for the preparation in a stool specimen derived from a clinical typical typhoid fever case furnishes the connecting bridge between cases 2 and 3, and again, the daily presence

of a physician in the house of patient 2 establishes beyond any doubt the incubation time of 12 days for case 3; this fact would therefore be additional proof of the correctness of the deductions above stated. Such conclusions, however, appear doubtful to the bacteriologist when he notices striking cultural and serologic differences in the offending organisms of cases 2 and 3, and it is only natural to suspect that case 3 was the result of an outside instead of an inside house contact infection. One of us (N. M. N.) reviewed epidemiologically in detail the various typhoid cases which were known to exist in the community, and which were thought to be reasonable sources of infection. It was not only possible to prove conclusively that the infection did not originate from any one of the outside cases, but the members of the household also unanimously agreed that Sch. had not taken any meals outside of his house during the illness of his daughter. While the bacteriologic findings suggest a new source of infection, all the epidemiologic data prove conclusively a contact infection. The importance of this fact will become apparent in connection with the discussion of the bacteriologic findings.

2. *Clinical Data.*—The clinical findings in case 1 were indefinite, and without a positive blood culture an early accurate diagnosis would have been impossible. Also in case 2 the course of the first febrile attack was mild and clinical diagnosis was only ventured during the relapse. It is not unlikely that this infection would have ended abortively had the patient remained in bed a few days longer. In neither case were rose spots observed, nor was the spleen definitely palpable. The Widal reactions were negative, and even in applying Dreyer's principle, exceedingly doubtful. Even the blood culture method, which, according to recent accounts of Eggerth,[39] is the most reliable procedure for the diagnosis of typhoid fever infections in the vaccinated, failed completely. A typical leukopenia and a slow pulse were, however, suggestive of such a disease. Stool and urine examinations of case 1 were negative. In case 2 one urine specimen contained irregular typhoid bacilli. Negative stool specimens were to be expected on account of the carbohydrate diet, which as Torrey[40] and we have repeatedly shown, reduces the viable B. typhosus to such a degree that a bacteriologic demonstration is frequently impossible even with brilliant green eosin plates.

[39] Jour. Infect. Dis., 1919, 25, p. 166.
[40] Ibid., 1915, 16, p. 72.

These observations confirm the facts that have been established by the medical service of the U. S. Army and recently presented by Soper [41] at the meeting of the American Public Health Association, namely, typhoid fever in the vaccinated may as a rule run a mild course and one difficult to recognize. Clinically, it is also impossible to differentiate a paratyphoid from a typhoid infection. It therefore could be suggested by our critics that in the light of the serologic tests the infections were caused by a nongas-producing B. enteritidis or B. paratyphosus. The clinical observations lend little support to this contention. The B. enteritidis infections thus far reported by Jochmann [42] and observed by one of us, are always abrupt and in their initial symptoms governed by marked gastro-intestinal reactions. Furthermore, relapses as Torrens and Whitington [43] and Jochmann have pointed out, are comparatively rare and usually of shorter duration than in true typhoid fever.

It may be a mere coincidence when Kisskalt [37] states in his summary that in laboratory infections the Widal reaction is frequently negative. Concerning this point accurate data determined by the macroscopic agglutination test should be collected from future cases.

The diagnosis of typhoid fever in the vaccinated was apparently connected with difficulties in the Army, otherwise a classification into (1) suspected (2) clinically and (3) bacteriologically proved cases would not have been advocated. Little comment is necessary when dealing with the clinical aspect of case 3; the symptoms and bacteriologic findings were typical in every respect.

3. *Bacteriologic Findings.*—Our bacteriologic findings with one cell cultures have established the following facts: Strain "I 75" and "Chr. 76" isolated from cases 1 and 2, respectively, behave as irregular, atypical typhoid bacilli. Strain 49 isolated from case 3 is a typical B. typhosus. The irregular strains exhibited the following variants: They are rapid dulcite, rhamnose and irregular arabinose fermenters; they are "blue typhoids" producing rapidly alkalies in bromcresol purple milk; one strain produced indol in the second generation; serologically, they are hyperagglutinable as living organisms and belong to a subgroup of group III of Hooker's typhoid classification. They are typhoid-inagglutinable in formalinized suspensions, but are as saprophytic strains agglutinated by B. enteritidis serums and by their

[41] Am. Jour. Pub. Health, 1920, 10, p. 301.
[42] Lehrbuch d. Infektionskrankheiten, Berlin, 1914, p. 85.
[43] Brit. Med. Jour., 1915, 2, p. 697.

own with immune serums. Typhoid immune rabbits are protected against the infection and intoxication by the irregular strain. These variants, with the exception of an acquired agglutinability for B. enteritidis serums, have remained constant in the course of at least 150 transplants on peptic digest agar. It is evident, that the irregular strains differed bacteriologically and serologically only in degree from the true B. typhosus. Inherent properties, like certain carbohydrate fermentations (rapid fermenters) and coreactions with B. enteritidis serums, are enhanced to a marked degree of activity, but there are no suggestions of true mutation, a conception which is well supported by the masterly analysis of the available facts in Eisenberg's [44] summary on "Bacterial mutation." This paper contains all the important references to atypical typhoid or paratyphoid strains, but we have been unable to identify our organisms with any one of the hitherto described irregular bacteria of the typhoid paratyphoid group. In many instances the method of identification has been so incomplete that it would be mere guess-work even to correlate the organisms recently described by Guerbet and Henry, Faroy, Lafforgue, Messerschmidt, Marotte, Fromme, Oette, Wagner, Goebel, Ohno, Wille, Broughton-Alcock [3] and others with our own.

Irregular fermentation reactions are frequently accompanied by similar variations in the serologic behavior. Inagglutinable and peculiarly receptive strains have been repeatedly described, but an analogue to our observation was not found in the references at our disposal. It is, however, emphasized that agglutinability is a variable characteristic (Henderson Smith [45]), and that it should be used with caution in the differentiation of closely allied variants of the typhoid-colon groups.

It would be interesting, indeed, to know how this intensification of the inherent properties was induced and how far the resultant variants influence the pathogenicity and epidemiology of the disease, and to what degree the occurrence of such variants influences our conception of the homogenicity of the B. typhosus group. Is it a mere coincidence that the progressively atypical and irregular strains occurred in two typhoid vaccinated persons and apparently the same strain reverted atavistically to a typical typhoid strain in an aged nonvaccinated man? We are not in a position to answer these questions, because test-tube experiments and innumerable rabbit and guinea-pig experiments have

[44] Ergebn. Immunitatsforschung, 1914, 1, p. 28.
[45] Trans. Fifteenth Int. Congress of Hyg. and Demogr., 1912, 2, p. 99.

not enabled us to accomplish this transformation. Laboratory animals are nonsusceptible to the B. typhosus, and even extended latency of this organism in the bile or tissues of rabbits, guinea-pigs, dogs or monkeys has in our experience created only inagglutinable but otherwise typical offsprings. A certain degree of adaptability to changes in the H-ion concentration of the substratum may be noted as was recently pointed out by one of us. But this fact has remained thus far the only tangible suggestion that existence in animal tissues may be conducive to the production of variants. The literature, however, contains sufficient observations which indicate that fermentative properties may be acquired on artificial mediums. B. typhosus can, as Twort,[46] Penfold,[16] R. Müller[15] and others have shown, gradually "mutate" into distinct variants, which, however are inconstant. After a series of cultivations on a substratum free from the enhancing carbohydrate fermentation stimulating substance, the variants as a rule revert to the original ancestral type.

It is quite plausible to assume that such a transformation of one or several true typhoid strains occurred in our case 1. In this connection we cannot overlook the possibility that perhaps animal paratyphoid strains to which the man was exposed underwent transformation. A change of their labile properties is in the realm of possibilities. Such a conception as well as the criticism that we were possibly working with mixed cultures can be readily dismissed. Old and recent observations have proved the low pathogenicity of animal strains of B. paratyphosus B and B. enteritidis, and again our discussion of the bacteriologic and serologic findings given above lend little support to this assumption. Repeated plating and finally the preparation of one cell cultures by the Burri method were chosen to rule out conclusively the danger of a mixed strain.

Passing through a second vaccinated host the irregular strain undoubtdly met an environment similar to the first, and it therefore preserved the new properties acquired. Only when flourishing in the tissues of an unvaccinated individual the variant reverted for reasons as yet unknown to the original typical B. typhosus. In our opinion, the most important question: "Is a change in metabolic activities also accompanied by alterations in pathogenic and antigenic properties?" cannot be answered conclusively. Animal infection and intoxication experiments fail to inform us concerning the true pathogenicity for

[46] Proc. Roy Soc., London, 1907, B. 79, p. 329.

man, and we are mainly dependent for an explanation on the results of our serologic tests. It was noted that strain "I 75" and "Chr. 76" differed in many respects from the type strain "Rawlings," and that these strains belong to a subgroup of the B. typhosus. It is the recognition of this fact that prompted this report and that furnishes some questions of considerable practical importance. The answers to some of these queries can perhaps be best introduced by the citation of a few lines from the recent book of Adami:[47]

> Still I shall feel that these pages have not been written in vain if I succeed in drawing increased attention to the fact that the bacteria are organisms acutely susceptible to changes in environment, that as species they are far from presenting constant characteristics, and that to a variability which may impress itself upon a greater or less number of generations is to be ascribed, in part, the differences between successive epidemics, between the successive stage of one epidemic, and between individual cases of disease.

We are well informed concerning the variability of the coccus group; but few authentic observations have thus far been made with micro-organisms of the typhoid group. This is in part the result of incomplete epidemiologic investigations and of abbreviated methods for the identification of the typhoid bacillus which will rarely disclose functional variants and therefore will never lead to the discovery of new types responsible for certain epidemics. As a matter of fact, the epidemiologist of today is frequently considered nothing more than an expert in detective and police methods, when his highest function primarily should consist of the intricate analysis of all the biologic and dynamic forces leading to an epidemic. For this reason he should have at his command a laboratory of his own, and a force of experts fully equipped to study biologically the suspected causes. With the progress of our methods of sanitation and preventive immunization the necessity for the detection of new disease producing variants should be proclaimed with unremitting insistence. Perhaps for no other disease is this demand more urgent than for typhoid fever. Only when these studies have been made on every occasion will it be possible to answer the mooted question: Does the prophylactic vaccination with a monovalent antigen really confer the maximum obtainable protection? It is not our intention to enter into a consideration of this controversy, but the observations of Mock,[2] Kisskalt[37] and our own clearly demonstrate that it is the vaccinated or the laboratory worker, who develops irregular typhoid strains. We should, therefore, insist on a most careful study of the bacterial strains from these sources.

[47] Medical Contributions to the Study of Evolution, New York, 1918, p. 131.

SUMMARY

This report describes the laboratory infections in a vaccinated caretaker (case 1) exposed to laboratory animals shedding living typhoid bacilli and a laboratory worker also immunized (case 2), who was regularly working with strains of the B. typhosus group. The last case apparently caused a severe and fatal house contact infection (case 3). From the blood of case 1 and the urine of case 2, on one occasion only, an irregular, atypical organism, and from the stool and urine of case 3 typical typhoid bacilli were isolated. The irregular typhoid strains ferment without gas production the usual carbohydrates, also dulcite, rhamnose and irregularly arabinose; they rapidly cause an alkaline reaction in milk, and develop small rhamnose papillae comparatively slowly. In formalinized killed suspensions they are only agglutinated by their own immune serums and recently also by B. enteritidis serum. As living organisms they are specifically clumped and sedimented by typhoid immune serums and can be classified by absorption tests with one of the subgroup of group III in Hooker's classification. Antiserums prepared with these organisms agglutinate typhoid bacilli in living suspension only and coagglutinate slightly, if at all, the representatives of the paratyphoid B or A group. As saprophytic strains they behave like nongas-producing strains of B. enteritidis from which group they cannot be separated even by careful absorption tests.

The possible bearing of these observations on the epidemiology, clinical aspect and bacteriology of typhoid fever is discussed, and it is suggested that special attention be paid to the occurrence and the detailed study of irregular typhoid strains in the typhoid-vaccinated.

A HEMOPHILIC, ANAEROGENIC PARACOLON BACILLUS FOUND IN A CASE OF INFECTED BILATERAL HYDRONEPHROSIS

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In the course of a systematic investigation of urinary infections primarily undertaken with the aim to reclassify by modern methods the various causative types and the subdivisions of the colon-paracolon group, we found an organism which was only cultivated on hemoglobin medium. This bacillus reverted atavistically to a saprophytic nongas-producing paracolon after artificial cultivation. A similar transformation of type took place in the urine of the host following vaccination and clinical recrudescence. The organism is here described and an account given of the case from which it was obtained.

History of the Case

A woman, aged 35, had always enjoyed excellent health up to a short time following her last childbirth. May, 1915. There was no history of focal infection such as teeth abscesses or tonsillitis. Following childbirth she had intermittent attacks of chills and fever which were attributed to an infection in the broad ligament of the left side. The urine was negative on examination and cultural study was not made. Later in May examinations of the urine showed a bacilluria due to a peculiar type of gram-negative (colon bacillus?) bacillus with a few polynuclear cells and a few hyaline casts. A blood culture was negative; spleen large and soft; marked hemorrhoids, not infected. The general poor condition continued; the hemorrhoids were troublesome and she complained of pain in the neck, principally in the back, and asthenia. In August, 1917, the urine still showed a slight trace of albumin with a number of white cells and bacteria. The patient was treated by rest in bed, regulation of diet, and control of the bowels in an attempt to relieve the urinary infection. In the latter part of 1917, the hemorrhoids were removed by operation. Complete gastro-intestinal studies made about this time were negative with respect to gallbladder or intestinal focus.

In June when the patient first came under our observation, the total phthalein output was 65% and bacteriologic study of the urine showed a gram-negative bacillus which grows only on blood agar plates. A stool specimen examined in June showed normal flora. Plain roentgenograms of kidneys, ureters and bladder were negative. A more complete urologic study later in June showed a condition of bilateral hydronephrosis with a tortuous ureter and movable kidney on the right side. On the left the hydronephrosis was apparently due to a valvelike obstruction at the ureteropelvic juncture. Infrequent attacks of fever with dull

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pain in the right or left side and severe headaches lasting for several days continued to recur. The patient was vaccinated during the months of July, August and beginning of September with a heat-killed suspension of the hemophilic organism and B. alkaligenes.

In Sept., 1918, ureteropyelography confirmed the previous finding and showed a rather dilated and tortuous upper right ureter and a hydronephrosis with a pelvic capacity of 40 c c and marked blunting of the minor calyces. The pyelogram of the left kidney was definitely square-shaped but its ureter was not dilated. The left pelvis had a capacity of approximately 45 c c. The separate phthalein output showed marked variation according to whether the tip of the ureteral catheter had entered the respective pelvis or not, as for example, on March 11, 1919, phthalein with the right catheter not in the right pelvis, but the left one well up in the pelvis appeared on the left side in 5 minutes, right side 8 minutes; the first 15 minute output, right side 2%, left side 15%, whereas, on March 14, with the position of the catheters reversed, the phthalein output on the right was 10% and on the left 5%. Total phthalein on March 13, 1919, was 30% first hour—second hour not taken. On April 10, total output was 27% first hour, and 11% second hour. Repeated phthalein estimations have shown a very definite and progressive diminution in renal function during the last 2 or 3 years.

The bacteriologic results with catheterized urine specimens obtained by us from June, 1918, to April, 1919, follow:

June 6, 1918: Bladder Urine: hemophilic organism, streptococci.

June 8, 1918: Bladder: hemophilic bacillus and B. alkaligenes. Right Kidney Urine: sterile, direct and enriched. Left Kidney Urine: hemophilic bacillus; enriched in plain broth sterile.

June 10, 1918: Bladder Urine: hemophilic bacillus and B. alkaligenes.

June 14, 1918: Bladder: hemophilic bacillus. Right Kidney: sterile direct; enriched in blood broth; hemophilic organism. Left Kidney: hemophilic bacillus.

June 18, 1918: Bladder: hemophilic bacillus. Right Kidney: sterile direct, enriched few cocci. Left Kidney: hemophilic bacillus and B. alkaligenes.

Sept. 15, 1918: Bladder direct: ∞ anaerogenic paracolon growing on nonhemoglobin mediums and B. alkaligenes in one drop of urine. Right Side: B. alkaligenes. Left Side: sterile, direct and enriched.

Sept. 19, 1918: Bladder: ∞ paracolon few B. alkaligenes. Right Side: few paracolon and innumerous B. alkaligenes.

March 11, 1919: Bladder: ∞ paracolon. Right Side: sterile. Left Side: ∞ paracolon.

April 26, 1919, a plastic operation on the left renal pelvis was made under gas and oxygen—Heineke-Mikulicz incision being made at the ureteropelvic juncture to enlarge the pelvic outlet. Culture of the urines obtained May 16 showed an entirely different type of infecting organism, namely, a pure colon bacillus, the typical organism previously found having entirely disappeared. The patient has been under observation since the operation to the present time and there has been no reappearance of the previous type of infection.

BACTERIOLOGIC FINDINGS. THE HEMOPHILIC ANAEROGENIC PARACOLON BACILLUS

The first specimen of catheterized urine collected in June, 1918, was plated in routine fashion on endo and sheep blood-veal agar plates; 4 c c of clear urine were also enriched in plain glucose broth. After 24 hours' incubation only 3-4 small, slightly reddish streptococcus colonies were observed on the endo-plates; the blood medium, however, was covered with innumerable discrete whitish

colonies not unlike those of B. influenzae. On closer inspection the isolated colonies were somewhat raised and very viscid on touch with a platinum needle. Microscopically, the growth consisted of short, stumpy or coccoid gram-negative bacilli. The enriched broth tubes were clear and showed only a faint, filmy sediment. Veal, peptic digest, casein and veal-ascitic fluid—or beef serum-agar plates with and without blood gave an identical result, namely, small colonies appeared only on the blood plates. Even after incubation for from 5-10 days no growth was visible. The phenomenon repeated itself, in the course of frequent cultures made from the urine of the bladder and ureters; as a rule the B. alkaligenes grew on all the mediums employed yet the predominant bacillus was only cultivated on a hemoglobin containing substrate.

Specimens of urine obtained on Sept. 15, 16, 19 and thereafter until March 11, 1919, gave, however, a very fine dewdrop-like growth of the originally strictly hemophilic organisms on ordinary plain or glucose or serum agar after 48-72 hours' incubation. On blood plates the growth was more profuse, the colonies were larger and somewhat darker in color. In the meantime, the strictly hemophilic strains having been kept on blood agar and tested at weekly intervals on plain agar, had acquired the property to grow fairly easily on blood-free mediums. On the average 10-12 transplantations on blood mediums were necessary to convert the parasitic, hemophilic type into a saprophytic one, which as such permitted a definite classification.

For the sake of clearness it appears advisable to describe collectively the findings on the parasitic strains isolated from June, 1918, until March, 1919, and those of the saprophytic strains as they developed either in the fresh specimen since Sept., 1918, or in the test tubes as a result of frequent transplantations. In this connection it may be stated that only purified cultures were investigated and that on several occasions 50 colonies of the hemophilic organism were tested on plain agar slants. Detailed studies were, however, made only with 5 representative offsprings of the parasitic and of the saprophytic colonies, respectively. The composition and reaction of the mediums were always identical and the differences between the two types of the same organism can therefore only be explained on the basis of an adaptation phenomenon. The characteristics of the strains are:

1. *Parasitic Strains:* Morphology: The urinary sediment of the left ureter as a rule gave a pure culture of the organism under consideration. Smears showed small coccoid-like bacilli which in shape and size resembled B. melitensis or certain forms of B. pseudo-influenzae; they were always immotile. In stained preparations they appeared as gram-negative short rods (0.3-1.5 mikrons), usually arranged in clusters. Some forms may show indications of a capsule. Material from cultures emulsified poorly but stained readily; the single organisms were generally in the first generation surrounded by a halo, suggesting a zooglea-like capsule, which was easily demonstrated tinctorially by treating the fixed smear with weak acetic acid. Some forms stained bipolarly and resembled in size B. coli. No flagella could be made visible. In old liquid cultures long filaments and other pleomorphic forms were constantly noted. The single organisms always appeared separated by a mucoid-intercellular substance.

Cultural Characteristics.—On blood plates small gray-whitish colonies developed readily inside of 24-36 hours under aerobic or semi-anaerobic environment. The bacillus was mesophile, the optimum temperature for growth was 37 C. At 22-25 C. occasionally a poor and slow growth was noticed. Under 20 C. no growth took place. In the course of a few days the colonies appeared

rather raised, conical, slimy and moist; they were very viscid and of mucous consistence. Occasionally threads of from 2.5 cm. in length could be easily withdrawn by touching them with a needle. The medium was not altered in color; there was no hemolysis. On blood plates and particularly on cooked hemoglobin mediums, a film-like, diffuse growth with a slight brownish discoloration in the butt was readily obtained. The water of condensation showed a stringy, slimy sediment. In blood broth a whitish sticky film covered the layer of red cells; after 6-8 days' incubation the hemoglobin was discolored and perhaps a slight turbidity of the supernatant broth was visible. The later reaction, being in our opinion the result of acid-split products, was particularly noted in glucose-blood broth tubes. Repeated attempts to grow the bacillus on various other hemoglobin free mediums failed. In sterile urine or urine broth no growth was obtained. Successive transplantations on blood agar produced a profuse saprophytic growth, which after 10-12 series was successively transferred to plain and to glycerin agar. The viability even on blood plants was only slight and weekly transfers to fresh mediums were necessary to keep the organism alive. Heating from 53-54 C. for 30 minutes sufficed to kill the organism when heavily suspended in salt solution.

2. *Saprophytic Strains.*—Morphology: Tinctorially and otherwise the strains differed in no respect from the hemophilic ones. When first isolated they fully possessed a distinct capsule which was, however, lost by artificial cultivation on plain agar; it was retained for about 10-20 generations on glycerol agar or broth.

Cultural Characteristics.—On peptic digest or glycerol or glucose agar plates seeded with urine obtained after the patient had been vaccinated with a suspension of the hemophilic bacilli, very small, streptococcus-like colonies made their appearance as a rule after 36-48 hours' incubation. In the course of 5-10 days these colonies increased slightly, acquiring a more slimy and raised appearance. The margins were sharp or slightly irregular. On glycerol agar the colonies were somewhat larger, markedly convex and very slimy; when touched with a wire loop the entire colonies were usually removed. Even on slants there was no tendency for spreading. The water of condensation had a slimy sediment. Broth cultures (glucose and glycerol) inoculated with the second or third generation showed occasionally a faint turbidity after 24 hours' incubation, which gradually disappeared in the course of another 24 hours; the tubes showed a clear upper part with a tenacious, slimy sediment. By shaking, a tuftlike formation was obtained, which remained for some time in the glycerol tubes the whole medium being very viscid. There was no gas, but a slight acid production in glucose broth. On Loeffler's serum and ascitic fluid agar a fairly thick, more or less compact viscid deposit was formed. On potatoes the recently isolated saprophytic strains failed to grow; the older strains cultivated artificially for over one year and six months gave a faintly visible film. Milk was not coagulated, but slightly acidified after from 4-6 days' incubation. Gelatin plate or stab cultures show after from 10-12 days small punctiform colonies with a finely granular inside structure. This medium is never liquefied, strains grown on glycerol or plain agar become more and more saprophytic and the cultures on the hand to day grow quite freely, but in comparison with B. coli less abundantly. Two strains under observation exhibit in this respect growth characteristics which resemble those of a stock culture of B. dysenteriae Shiga. In liquid mediums and in brain suspensions the bacteria will remain viable for at least 2 to 3 weeks.

Some of the nonhemophilic strains produced indol in the second and third generation. Highly saprophytized strains have failed to give this reaction in

various peptones (Witte's, Difco, Parke, Davis and Co.). Lead acetate and neutral red remained unchanged; the methyl red test was positive, the V/P reaction was negative. The strains tested quite recently were more brilliant green tolerant than B. coli or B. dysenteriae. Based on the above cited characteristics the group number of our bacillus is B. *222.2332033.*

The fermentation reactions, namely, production of acid, noted in Hiss serum water or peptone-phosphate solutions, are summarized in table 1. For comparison two anaerogenic colon strains, also isolated from urinary infections, are included.

TABLE I

THE CULTURAL CHARACTERISTIC OF ANAEROGENIC STRAINS ISOLATED FROM URINARY INFECTIONS

	Hemophilic Anaerogenic Paracolon	Anaerogenic Metacolon	Anaerogenic B. Coli
Motility	0	0	±
Gelatin liquefaction	0	0	0
Milk	Alkaline or acid P_H 6.6 (10 days)	Alkaline	Acid—coagulated (5 days)
Indol	±	±	+
Lead acetate	0	0 or +	0
Neutral red	0	0 or +	+
Glucose	A. P_H 5.5 (5 days)	A. P_H 5.8	A. P_H 4.8
Levulose	A.	A. P_H 5.5	A.
Galactose	A.	A.	A.
Mannose	A.	—	A.
Mannitol	A. P_H 5.8 (5 days)	0	A. P_H 4.8 (5 days)
Maltose	A.	0	A.
Rhamnose	A.	0	0
Xylose	A.	0	A.
Arabinose	A.	0	A.
Sorbitol	A.	0	A.
Dulcitol	0	0	A. and 0
Adonitol	0	0	0
Lactose	0	0	A.
Sucrose	0	0	0
Raffinose	0	0	0
Erythrite	0	0	0
Salicin	0	0	A.
Dextrin	0	0 or A. slight	0
Inulin	0	0	0
Inosite	0	0	0
Glycerin	0	—	—
Voges-Proskauer	0	0	0
Methyl red	+	+	+
Urine	Alkaline (15)	Alkaline	Acid

In this connection we emphasize the fact that only saprophytic strains, after ten successive transplantations on plain agar were tested, the exact nature of the organism being recognized only at a period when all the original hemophilic strains had acquired saprophytic properties. The tests were repeated recently with the strains kept under artificial cultivation for over a year and results identical with those noted September, 1918, and August, 1919, were obtained. Acid, but no gas is formed by our bacillus in mediums containing the various hexoses, mannitol maltose, rhamnose, xylose, arabinose and sorbitol, but not in dulcitose, adonitol, sucrose, raffinose, salicin, dextrin, inulin, inosite and

glycerin. The acid fermentation is rather sluggish and the end reaction never goes below P_H 5.5. These characteristics would place our organism with group III of the classification of Winslow, Kligler and Rothberg; some reactions, however, indicate a relation to group IV.

Agglutination Tests.—The cultural characteristics cited above, suggested the agglutination reactions to place this organism more satisfactorily in the dysentery-paratyphoid group. It was immediately realized that such a procedure would be of limited value on account of the capsulated nature of the bacteria. The negative results obtained with a variety of specific serums testing the capsulated parasitic and saprophytic strains, are therefore of no significance. Even the serum of a rabbit highly immunized with such strains failed to agglutinate the immunizing bacillus. As already stated, the parasitic strains gradually lose their capsules and it is with such organisms that a new series of agglutination tests was set up. Again, negative results were recorded. A rabbit-immune serum with a titer of 1:400 agglutinated only several strains of our bacillus. A large series of anaerogenic paracolon and colon strains were clumped in dilution of 1:2 or 1:5. The same strains were, however, also agglutinated by normal rabbit serums in the same dilutions.

The negative agglutination test obtained with the patient's serum must be ascribed to the use of a capsulated organism and cannot serve as a criterion for the nonpathogenicity of this paracolon bacillus. With the saprophytic noncapsulated strains additional tests were only possible at a time when the urinary flora had been displaced by a typical B. coli communior. In this connection we desire to call attention to the importance of serologic studies in urinary infections. It is not uncommon to find negative agglutination reactions[1] in chronic pyelitis and cystitis, and even after a prolonged, intensive vaccination that may result in complete recovery such tests can be entirely negative. Observations of this character have more than academic interest and should therefore be investigated in detail.

Pathogenicity.—Young guinea-pigs and mice inoculated intraperitoneally with $\frac{1}{10}$ slant of a blood-agar culture of the parasitic strain may die in from 24-48 hours showing at necropsy a muco-fibrino-purulent peritonitis. Subcutaneous injections produce a slight infiltration. Rabbits on intravenous application tolerate one-half and even one slant of the same organism. The saprophytic strains are only fatal for guinea-pigs in 1-2 slant doses. Other animals were not used for pathogenicity tests. A specific localization in the urinary passages of rabbits was not noted in a small series of experiments. Old cultures contain apparently toxic substances, which produce on intravenous inoculation of rabbits transitory illness; rhinitis, rapid respiration and diarrhea are usually observed for from 10 to 12 hours.

Intravesicular injections of rabbits with parasitic and saprophytic strains failed to produce a cystitis; the introduced organism could be demonstrated for from 24-72 hours.

B. alkalignes.—The concomitant B. alkaligenes regularly found in the urine from the bladder or from the left or the right ureter corresponded in every respect with the type strain at our disposal. Culturally the colonies of this organism were characteristic and could be distinguished from the anaerogenic, hemophilic paracoli. Transformation of properties, which could suggest a close relation with these organisms, were not recorded. The strain was non-

[1] See Dudgeon: Lancet, 1908, 1, p. 615.

pathogenic for rabbits in doses of 1/5 of a slant and did not exhibit specific elective properties for the rabbit urinary system. The patient's serum agglutinated the organism on June 20, 1918, in a dilution of 1:80. This reaction may be interpreted as a slight response to the invasive and pathogenic properties of this organism. Unfortunately no opportunity was afforded to test the patient's serum after vaccination or later in the course of her illness.

As stated in the history, the bacterial flora changed completely after the operation. The anaerogenic, originally also hemophilic, paracolon has disappeared; a typical B. coli communior has always and repeatedly been isolated. Many tests have uniformly demonstrated an organisms that behaved biochemically and serologically in an identical manner and characteristic of B. coli.

DISCUSSION

We carefully consulted the extensive literature [2] on the bacteriology of urinary infections. Most of the publications are valueless, the data being collected in a period when blood plates or enrichment in blood broth were not considered necessary as a routine procedure for the study of urinary micro-organisms. Due credit should be given to D. J. Davis,[3] who in 1910 called attention to the occasional occurrence of hemophilic bacilli in urine; also to V. C. David [4] who in a series of 50 urinary cultures derived from diseases of the bladder and kidneys encountered a gram-positive, slightly anaerobic influenza-like bacillus. At first we were inclined to consider the parasitic strain of our bacillus closely related to the one described by Davis. His organism grew only on hemoglobin mediums, the colonies were very minute and opaque and were always hemolytic. The latter features were not observed with our organism; and again the size and tendency for thread formation were more colon-like than diptheroid, as carefully described by Davis for his organism. In many respects our bacillus resembled the B. pseudo-influenzae isolated by Wolff [5] from the bronchi of a rat. Our hemophilic organism, in a manner similar to Wolff's bacillus after repeated transplantations on artificial medium or in the human host subsequent to vaccination, to a clinical relapse or to other unknown factors, reverted "atavistically" to a saprophyte, comparatively easily cultivated on ordinary hemoglobin-free medium. Deprived of its hemophilic tendencies

[2] Rovsing: Die Infektions-Krankh. der Harnwege, 1899; Koll: Intern. Abstr. of Surgery, 1915, 20, p. 349; Franke: Ergebn. d. Chir. u. Orthopädie 1913, 7, p. 671; Blumenthal and Hammer: Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1908, 18, p. 642; Kodama and Krasnogorski: Centralbl. f. Bakteriol. I, O., 1913, 69, p. 8; Wulff: Centralbl. f. Bakteriol. I, O., 1912, 65, p. 27.

[3] Jour. Infect. Dis. 1910, 7, p. 599.

[4] Surg., Gynec. & Obst. 1914, 18, p. 432.

[5] Centralbl. f. Bakteriol., I, O., 1903, 33, p. 407.

the bacterium could be readily studied by means of carbohydrate mediums and some suggestions as to its possible classification were obtained. Applying the recent findings of Winslow, Kligler and Rothberg,[6] Gettings and Inman [7] and others made with this class of organisms, one would be inclined to place the saprophytic strains with the B. Flexner-dysenteriae or B. gallinarum group of bacteria. The agglutination and pathogenicity tests and the viscid appearance of the colonies did not, however, justify this conclusion, irrespective of the fact that Foerster [8] and also Hilgers [9] quite recently described the findings of true agglutinable dysentery bacilli in the urine. It is not unlikely that the organisms described by the two workers belong to the same group of bacteria as our own. Decidedly more suggestive are the descriptions given by Herrold and Culver [10] for the so-called "paracolon-bacilli." The few carbohydrate reactions recorded by them correspond well with those found for our bacillus. Nongas-producing urinary colon, paracolon or even meta-colon (Jensen-Bahr's classification in Wulff's publication) are not infrequently encountered in urines. Mair,[11] Wilson,[12] Sörensen [13] and Arkwright [14] described such organisms and we therefore decided to place our bacillus tentatively with the paracolon group and designate it as an anaerogenic paracolon bacillus.

The peculiar mucoid, viscid character of the colonies of the saprophytic strains recalled the observations by one of us (K. F. M.)[15] made several years ago on a bacillus (B. nephritidis-equi or B. viscosum-equi of Magnusson [16]) isolated from renal abscesses of a horse. This organism produced such a slimy zooglea-like growth that filaments of from 10 to 20 cm. could be withdrawn on touching with a needle. B. viscosum is a colon-like organism; it grows only on glycerol agar medium, produces a toxin and dies out readily on artificial mediums. Degen [17] found a similar organism, which he described under the name of B. polymorphus-suis. Unfortunately the descriptions available are

[6] Jour. Bacteriol., 1919, 4, p. 429.
[7] Medical Research Committee, Special Report Ser. No. 30, 1919.
[8] München. med. Wchnschr., 1918, 65, p. 205.
[9] Centralbl. f. Bakteriol., I, O, 1919, 83, p. 414.
[10] Jour. Infect. Dis. 1919, 24, p. 114.
[11] Brit. Med. Jour. 1906, 1, p. 438.
[12] Jour. Hyg., 1908, 8, p. 543.
[13] Centralbl. f. Bakteriol. I, O., 1912, 62, p. 582.
[14] Jour. Hyg., 1913-1914, 13, p. 68.
[15] Report of Government Bacteriologist; Dept. of Agriculture, Pretoria, Transvaal, 1908 1909, p. 122-158.
[16] Jour. Comp. Pathol. & Therap. 1919, 32, p. 143.
[17] Thesis, Giessen, 1907.

incomplete and it would be unwise to consider our bacillus identical with one of these organisms even when some of the characteristics seem to be analogous.

The systematic position of our bacillus is of subordinate interest when we consider more carefully the observation dealing with what was termed parasitic and saprophytic strains. Originally isolated from the urine of a case of hydronephrosis, the bacillus exhibited strict hemophilic properties and distinct capsule formation. These characteristics were subsequently lost on artificial cultivation in vitro and apparently also in the human host. It may be mere coincidence that our bacillus, which originally grew on blood only, developed on plain agar when seeded with urine collected from the patient after she had been vaccinated and had suffered from a relapse. One fact is certain, that systematic urine cultures made this observation possible and materially assisted in the final identification of the organism.

Hemophilic and anaerogenic properties and capsule formation are suggestive, in the sense of Sauerbeck, of a "bacterial immunity by structural adaptation." As already suggested, certain cultural characteristics such as viscosity and loss of gas production are not uncommon with organisms obtained from urinary infections. We have been consulted repeatedly concerning such non-gas producing, slow lactose fermenting colon bacilli isolated from the urine, and we gained the impression that possibly one or several factors as yet incompletely understood or investigated exert a strongly modifying influence on the microbes of the urinary tract. Only in assuming such a condition is it possible to appreciate the fantastic list of bacteria described about 10 years ago by Tanaka.[18] In our particular case the adaptation of our bacillus was not only directed against these rather common influences, but was primarily intended for existences in living tissues. Capsule formation and preference for hemoglobin substrata made their appearance. Removed from the soil to which the organism had been functionally adjusted, and grown on artificial mediums, it gradually reverted atavistically to its ancestral type, namely, a nongas-producing paracolon. It is not unlikely that in the course of time and on suitable mediums, our organism may even acquire the ability to produce gas from some carbohydrates. Such a transformation has been described by Arkwright for a nongas-producing B. acidi-lactici isolated from the urine. And again

[18] Ztschr. f. Urol., 1909, 3, p. 5.

Revis[19] and Penfold[20] were able to suppress gas production by the use of chemical—malachite green and chloracetic acid. How far the concomitant B. alkaligenes influenced the adaptation of the parasitic strains and how far the vaccination and the frequent clinical recrudescences favored reversion to type cannot be answered definitely. In a paper on irregular typhoid bacilli recently written by one of us (K. F. M.)[21] attention was called to the importance of functional changes of micro-organisms causing infections in immunized or protected human beings. There are sufficient observations available that clearly indicate that the urinary secretion may exert a "degenerative" or inhibitive influence on the functions of many organisms of the colon group. Studies on bacteria isolated from urinary infections should therefore offer interesting material to the important problem of bacterial variability and adaptation.

SUMMARY

A bacillus isolated from the urine of a case of bilateral infected hydronephrosis is described. It grew as a parasitic capsulated strain only on a medium containing hemoglobin. The bacillus is apparently a member of the paracolon group of bacteria found in urine and is best designated as hemophilic nonaerogenic paracolon. After four months' cultivation artificially it acquired the property of growing on hemoglobin-free substrata and fermented without gas production the following carbohydrates: hexoses, mannitol, maltose, rhamnose, xylose, arabinose and sorbitol. Samples of urine cultured after the patient had been vaccinated with a heat killed, tricresolized bacterial suspension of the original parasitic strain and after a clinical recrudescence, demonstrated the same organism which grew on nonhemoglobin mediums and which in every respect corresponded with the saprophytic strains obtained by successive cultivation on artificial mediums.

[19] Centralbl. f. Bakteriol, II, 1911, 31, p. 1.
[20] Jour. Hyg., 1912, 12, p. 195.
[21] Jour. Infect. Dis., 1920, 27, p. 46.

OPSONIC REACTIVATION OF ANTIPNEUMOCOCCUS SERUM

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Mennes [1] first noted that the serum of animals immunized against pneumococci contains substances that favor phagocytosis of virulent pneumococci. Wright and Douglas [2] described as opsonins substances in normal serum that attach themselves to bacteria and prepare them for phagocytosis. Neufeld [3] showed that immune serum may alter bacteria so that they become susceptible to phagocytosis, and he called the substances responsible for this change bacteriotropins. According to Hektoen,[4] there appears to be no fundamental difference between opsonins and bacteriotropins; normal serum contains a variety of opsonic substances each of which is specific for a given bacterium, and in immune serum the specific opsonic substance for the particular bacterium or cell with which immunization has been produced is developed in a high degree; in both cases the full effect is produced by the interaction of two substances, one of which is destroyed by heating, while the other remains unaffected; the latter is opsonic in itself but its action is enhanced by the heat sensitive element, and it is the heat resistant element that is produced specifically by experimental immunization or as the outcome of spontaneous infection. Clough [5] found that soon after the crisis in pneumonia and also after lysis, the serum may be actively opsonic for highly virulent homologous pneumococcus. He states [6] that when the serum of patients recovering from pneumonia "became inactive or feeble as a result of overheating or dilution, the phagocytic activity could not be restored or increased by the addition of complement."

The purpose of my experiments was to study once more the opsonic activity of antipneumococcus serum with special reference to this problem of reactivation.

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[1] Ztschr. f. Hyg. u. Infectionskrankh., 1897, 25, p. 413.
[2] Proc. Royal Soc., 1903, 72, p. 857.
[3] Deut. Med. Wchnschr., 1904, 30, p. 1458.
[4] Jour. Am. Med. Assn., 1914, 62, p. 254.
[5] Bull. Johns Hopkins Hosp., 1913, 24, p. 295.
[6] Ibid., 1919, 30, p. 167.

The pneumococci used in the experiments were obtained from the Army Medical School. The types 1, 2 and 3 strains are virulent, about one ten millionth c c of an 18-hour broth culture killing mice within 48 hours. The type 4 strain (74), also from the Army Medical School, was derived from a case of influenza pneumonia. The cultures were transferred on blood-agar slants every third or fourth day. For the experiments a 24-hour growth in 0.2% glucose broth was used.

Antipneumococcus serum of types 1, 2 and 3 was also obtained from the Army Medical School. The type 4 serum was the serum of a rabbit immunized against strain 74. Serum from six cases of lobar pneumonia was studied also, blood being drawn from the vein at the elbow 4-6 days after crisis. In one case (patient 2) blood was withdrawn the first day after the crisis.

Human leukocytes were used: About 1 c c of blood was taken from the lobe of the ear of a normal person and mixed with 2% solution of sodium citrate; after centrifugation and washing with 0.85% salt solution, the leukocytic layer was removed and suspended in salt solution. Fresh normal human serum was obtained in the usual way.

The opsonic activity of the serums was determined by mixing serum, salt solution, leukocytes and bacterial suspension in capillary pipets, which were incubated for 30 minutes at 37 C., smears made, and stained with carbol thionin. The degree of phagocytosis was estimated by counting the number of leukocytes per 100 that showed phagocytosis and also the number of pneumococci ingested by each leukocyte. In the activation experiments, the same process was followed except that about 0.01 c c of the fresh (activating) serum was added to the mixture, the amount of salt solution being reduced accordingly.

Pneumococcus type 1 proved resistant to phagocytosis with fresh normal human serum, the greatest number of leukocytes showing phagocytosis in any one specimen being 8 per 100 and the average number of bacteria in each phagocytic leukocyte being 2. Pneumococcus 2 also was resistant to phagocytosis with fresh normal human serum, the greatest number of leukocytes showing phagocytosis in any one experiment being 3 in 100. Pneumococcus types 3 and 4 were more readily phagocyted under the influence of fresh normal human serum than the strains of types 1 and 2.

Antipneumococcus serums types 1, 2 and 3 were found to have only slight opsonic action on the homologous pneumococci, being even less active than fresh normal human serum. This was particularly true of

serum 3. Serum 4 was actively opsonic for pneumococcus 4. Although the opsonic activity of serums 1, 2 and 3 was very slight, agglutination occurred in all the preparations, and in pneumococci tended to group themselves about the leukocytes.

The failure of the antipneumonic serum to opsonize homologous pneumococci may be explained by the absence of the thermolabile, complement-like element, and in that case the addition of fresh normal human serum should result in an increase in phagocytosis of the pneumococci. Accordingly, a number of activation experiments were made and, as shown in table 1, a marked increase in the action of antipneumonic serum, type 1, for the homologous pneumococcus resulted after the addition of a minute quantity of fresh normal serum. Serums 2 and 3 failed to show any increase in opsonic activity, after the addition of fresh normal human serum; serum 4, while relatively active, was distinctly enhanced in action by the addition of normal serum.

TABLE 1
REACTIVATION OF ANTIPNEUMOCOCCUS SERUM, TYPE 1

Antipneumococcus Serum	Pneumococci (Broth Culture)	Leukocytic Suspension	Normal Human Serum	Salt Solution	Results: Percentage of Phagocytic Leukocytes	Results: Number of Pneumococci per Active Phagocyte
0.1	0.1	0.1	—	0.1	8	2
0.1	0.1	0.1	0.01	0.09	44	4
0.01	0.1	0.1	0.1	0.09	47	5
0.01	0.1	0.1	—	0.19	6	1
—	0.1	0.1	0.01	0.19	5	1
—	0.1	0.1	0.1	0.1	4	1

TABLE 2
REACTIVATION OF HEATED POSTCRITICAL PNEUMONIA SERUM FROM PATIENT 3

Antipneumococcus Serum	Pneumococci (Broth Culture)	Leukocytic Suspension	Normal Human Serum	Salt Solution	Results: Percentage of Phagocytic Leukocytes	Results: Number of Pneumococci per Active Phagocyte
0.1 unheated	0.1	0.1	—	0.1	50	4
0.1 heated	0.1	0.1	—	0.1	1	4
0.1 heated	0.1	0.1	0.01	0.19	36	6
0.01 heated	0.1	0.1	0.1	0.09	12	3
—	0.1	0.1	0.1	0.1	4	1

Pneumococcus, types 1 and 3 already described, were used to determine the opsonic activity of serums of 6 pneumonia patients after crisis. Of the 6 serums, 5 proved to be actively opsonic and agglutina-

tive for pneumococcus type 1, but not for type 3. The serum of patient 2 failed to cause phagocytosis of either type 1 or 3. The occurrence of such distinct agglutination and phagocytosis of pneumococcus type 1 suggests that it concerned a type 1 infection in 5 of the cases.

The serums were heated for 30 minutes at 56 C., and the opsonic power was greatly reduced thereby, but on the addition of a small quantity of fresh normal serum to the heated serum, the opsonic activity was restored almost to its original strength, as illustrated in table 2.

SUMARY AND CONCLUSION

The results show that the opsonic power of the samples studied of antipneumococcus serum of types 1 and 4 was increased by the addition of a minute quantity of fresh normal human serum; that serum from 5 patients with lobar pneumonia after crisis was actively opsonic for a pneumococcus strain of type 1, but lost this power in large measure on being heated to 56 C., regaining it, however, almost wholly by the addition of small quantities of fresh normal human serum in itself but feebly opsonic. The conclusion is that opsonin in antipneumococcus serum can be reactivated.

ON THE TOXIN FOR LEUKOCYTES PRODUCED BY STREPTOCOCCI (STREPTOLEUKOCIDIN)

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Ruediger[1] found that the filtrates of virulent streptococcus cultures might suspend the destruction of nonvirulent streptococci by leukocytes. A little later, from observations on the appearance of leukocytes during phagocytosis in vitro, Hektoen[2] made this statement:

So far as our observations have extended centrifugated culture fluids of virulent streptococci, staphylococci and anthrax bacilli reduce phagocytosis of the corresponding nonvirulent strains, principally, I believe, through direct action on the leukocytes, because the fluids in question have but a comparatively slight inhibitive or destructive effect on the opsonin in normal serum, but reduce greatly by one hour's contact the phagocytic power of leukocytes with respect to previously sensitized bacteria. In full harmony with this loss in phagocytic power are the marked functional and morphologic disturbances in the leukocytes placed in fluid containing the products of the growth of virulent bacteria. Thus, when leukocytes are placed in the culture fluids of a virulent streptococcus and examined from time to time on the warm stage, they are seen to lose ameboid movement and to swell greatly in from 30 to 60 minutes, whereas the leukocytes in control experiments remain normal in form and motion several times as long."

M'Leod[3] noted a leukocidal effect of certain streptococci in rabbits in vivo, but he did not succeed in demonstrating any leukocidal action with streptococcal filtrates.

From these observations it may be concluded that virulent streptococci or their culture fluids may have an unfavorable action on leukocytes, but the subject apparently has not been studied systematically.

BACTERIAL LEUKOCIDIN

Generally speaking, the production by bacteria of substances that are toxic for leukocytes has received little attention with the exception of the leukocidin produced by staphylococci. Van de Velde[4] injected clear filtrates of 24-hour cultures of Staph. aureus in the pleural cavity of rabbits and found that the leukocytes changed, the nuclei becoming distinct and ameboid movement lost. When leukocytes were added to the filtrate in a test tube and left in the incubator for 24 hours, the same result was obtained. He named this toxic sub-

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[1] Jour. Am. Med. Assn., 1905, 44, p. 198.

[2] Ibid., 1906, 46, p. 1407.

[3] Jour. Path. & Bacteriol., 1915, 19, p. 392.

[4] LaCellule, 1894, 10, p. 403.

stance leukocidin. Borrissow[5] placed small capillary tubes filled with bacteria-free culture fluids of Staph. aureus, under the skin of rabbits and dogs, and noted that leukocytes not only were attracted by the fluid, but were also destroyed. When he left the tube subcutaneously in a dog for four days, many broken-down leukocytes were found in the fluid.

According to Deny and Van de Velde,[6] staphylococcus leukocidin is a metabolic product that acts not only on leukocytes, but on other cells, such as connective tissue cells and the cells of the sympathetic nervous system. They were not able to demonstrate the presence of any leukocidin when they injected into the pleural cavity, colon, diphtheria or typhoid bacilli or pneumococci.

Bail[7] cultivated virulent Staph. aureus in 40.0 c c of 1% glycerol with 15 c c of rabbit serum, and in 80 c c of broth with 20.0 c c of the fluid of ovarian cysts. In either medium the largest quantity of leukocidin was realized in cultures which had stood for 11 days. He injected into the pleural cavity of rabbits of about equal size and weight, from 7.0-12.0 c c of a germ-free aleuronat suspension, and after 24 hours he injected into the same place from 1.0-3.0 c c of filtrate containing leukocidin. When unheated filtrate was injected, the leukocytes degenerated in the course of from 1-1½ hours, but later normal leukocytes appeared in large quantity, the leukocidin by this time having disappeared.

Georgiewski[8] states that the culture fluid of B. pyocyaneus was injurious to leukocytes and that antipyocyaneous serum did not neutralize this effect.

Eisenberg[9] also found that in staphylococcus filtrates leukocytes rapidly become rounded, and the protoplasm homogeneous, losing its granules; the crescent-shaped nuclei were dissolved or fused into one lump.

In the study of leukocidin, the work of Neiser and Wechsberg[10] is especially notable. They skillfully made use of the reducing power of leukocytes in their study of the production of leukocidin by staphylococci. According to their results, when staphylococci are cultivated in an alkaline medium, leukocidin appears after four days, and after one week the production reaches its maximum. The amount of leukocidin depends on virulence, the strains of greater virulence producing a larger amount. The amount of leukocidin may be increased if the virulence is increased by passage through the animals, in the case of strains that have been grown artificially long enough to lose their virulence. They found that this leukocidin was destroyed by heating at 56 C. for a short time, and that the normal serum of horse and man contains antileukocidin which could be produced also by immunization.

Thus it appears that the leukocidin production by staphylococci has been studied fairly completely by several authors, but the production of leukocidin by streptococci and other bacteria has not been studied so thoroughly.

TECHNIC

In this work on the leukocidin produced by streptococci, I have used the same medium that I used in the study[11] of streptolysin,

[5] Ziegler's Beiträge, 1894, 16, p. 432.
[6] LaCellule, 1895, 11, p. 395.
[7] Arch. f. Hyg., 1898, 32, p. 133.
[8] Ann. de l'Inst. Pasteur, 1899, 13, p. 298.
[9] Comp. rend. Soc. de Biol., 1907, 62, p. 491.
[10] Ztschr f. Hyg. u. Infektionskr., 1901, 36, p. 299.
[11] Jour. Infect. Dis., 1919, 25, p. 509.

namely, broth (plain or 0.2% glucose) containing serum in a 1 : 9 dilution. The filtrates (Maasen) of cultures of streptococci in this medium may contain substances that act as toxins for leukocytes, and in this article active filtrates are designated simply as leukocidin or streptoleukocidin.

Leukocytes were obtained in two ways: First, by injecting aleuronat into the pleural or abdominal cavity of animals: 8% aleuronat is sterilized by boiling for 30 minutes, suspended in 0.9% salt solution and cooled to the body temperature. The amount to be injected depends on the size of the animal; in rabbits 8.0-10.0 c c of aleuronat suspension may be injected into the pleural cavity, and 20.0-30.0 c c into the abdominal cavity. After from 8-15 hours the animal is bled to death in order to prevent the inflow of blood into the exudate; then the pleural or abdominal cavity is opened and the exudate withdrawn by means of a pipet. In order to prevent coagulation an equal amount of 1.5% sodium citrate is added.

The second method is generally applicable, but especially when aleuronat injection cannot be made: Into a 1.5% solution of sodium citrate in 0.9% salt solution, from one-half to one-third the volume of blood is introduced, and after shaking well to prevent coagulation, the mixture is centrifugated. The fluid above the corpuscles is removed and replaced with an equal volume of 0.9% salt solution, when centrifugation is repeated. This process is again repeated twice or three times in order to free the cells from serum and citrate. After the final centrifugation the thin, white film, which contains comparatively numerous leukocytes, on top of the red corpuscles is removed carefully, mixed with 0.9% salt solution and used in the experiments.

Tests for Leukocidin.—The Microscopic Method: A mixture of the fluid to be tested for leukocidin and leukocytic suspension is examined microscopically on a warm stage. When leukocytes are placed in suitable streptococcus culture fluid and examined from time to time on the warm stage they are seen to lose their ameboid movement and to change their appearance; small transparent vesicles may appear in the cells; the nucleus becomes irregular, and granules may gather in the leukocyte which finally may be dissolved.

2. The Bioscopic Method: Ehrlich observed that living cells require a certain amount of oxygen which they appropriate from their surroundings. This ability of the living cell to cause a reduction is the basis of the bioscopic method. The addition of an easily reducible dye to a suspension that contains leukocytes offers a means to determine

whether the leukocytes are living or dead. I have used methylene blue, prepared according to the following formula; methylene blue, 1.0 c c; absolute alcohol, 20.0 c c; distilled water, 29.0 c c.

The first step in a given case is to determine the minimum quantity of leukocytic suspension which causes a reduction of methylene blue. Different densities of leukocytic suspension are made with 0.9% salt solution and the mixture made up to 2.0 c c. Two drops of methylene blue solution are added. The mixture is covered with a layer of liquid paraffin in order to prevent reoxidation by the air. The tubes are placed in an incubator at 37 C. for two hours. Small test tubes about 0.7 cm. or so in diameter are used. If reduction occurs the solution becomes colorless, if no reduction occurs the color remains green.

The second step is to determine the effect of the supposed leukocidin. To twice the minimum quantity of leukocytic suspension causing reduction as determined, different quantities of leukocidin fluid are added and each suspension diluted to 2.0 c c with 0.9% salt solution. These mixtures are incubated for 1½ hours at 37 degrees; at the end of this period two drops of methylene blue solution are added and covered with a layer of liquid paraffin; again they are incubated for 2 hours when the readings are made. If the tubes retain a green color it indicates that something has killed the leukocytes and prevented reduction. If the mixtures are colorless, that is, if reduction has taken place, there has been no lethal effect on the leukocytes. In the tables the results are given as positive (+ or ±) when the reduction of methylene blue by the leukocytic exudate was prevented by the culture filtrate; that is, when leukocidal effect was demonstrated; otherwise as negative (0).

LEUKOCIDIN PRODUCTION BY VARIOUS STRAINS OF STREPTOCOCCI

By means of the methylene blue reduction test I have examined a number of different strains of streptococci for the production of leukocidin in serum broth cultures. As a general rule, streptococci, hemolytic as well as nonhemolytic when nonvirulent and easily phagocytable under the influence of normal serum, do not produce any leukocidin demonstrable by the reduction test. Virulent strains, however, produce leukocidin in demonstrable quantities; such strains are not as a rule phagocytable under the influence of normal serum. Most of my experiments have been made with a typical hemolytic streptococcus (Str. pyogenes) originally isolated from a human abscess and subjected

to several passages through rabbits as well as mice, being of a rather high degree of virulence for these animals. Of the original cultures of green-producing streptococci examined for leukocidin production, a strain from the lung in a case of influenzal bronchopneumonia produced considerable leukocidin. The other strains examined gave negative results except for traces in the case of a coccus isolated from the blood in measles and of another coccus obtained from the brain after death from influenza. I may say also that my results indicate that certain typical pneumococci may produce leukocidal substances, but I have not made a careful study of such production by pneumococci.

The fact that animal passage raises the power of hemolytic streptococci to produce leukocidin is illustrated in table 1.

TABLE 1

VIRULENCE OF STREPTOCOCCI AND LEUKOCIDIN PRODUCTION

Culture Filtrate	Exudate	Results	
		Original Culture	Culture after Three Rabbit Passages
1.0	0.6	+	+
0.75	0.6	+	+
0.5	0.6	0	+
0.25	0.6	0	+
0.125	0.6	0	+
0.05	0.6	0	+
0.025	0.6	0	0
—	0.6	0	0
0.5 (heated to 60 C. for 30 minutes)	0.6	0	0

+ = leukocidin present. 0 = — no leukocidin present.

ACID FORMATION AND LEUKOCIDIN PRODUCTION

It is known that streptococci form acid in cultures. At the time when the existence of streptolysin itself was in doubt, it was suggested that hemolysis by the culture fluid of streptococci was due to the acid in the fluid, but the work of Hellens,[12] Sekiguchi,[13] and others, have clearly shown that there is no relation whatever between acid formation and streptolysin. Furthermore, my experiments indicate that acid formation and leukocidin production do not go hand in hand. According to observations made at various intervals, the amount of acid formed is either stationary or increases in the culture fluid (serum and plain or 0.2% glucose broth, 1:9), after from 48-72 hours, when the amount of leukocidin is found to decrease (table 3). Therefore, it seems clear that there is no close relation between the production

[12] Centralbl. f. Bakt., I, O., 1913, 68, p. 692.
[13] Jour. Infect. Dis., 1917, 21, p. 475.

of leukocidin and acid formation. But it appears that there may be a more definite relationship between the production of a leukocidin and streptolysin, for when streptolysin. is at its maximum, leukocidin is also at its maximum.

LEUKOCIDIN PRODUCTION IN BROTH WITH BLOOD SERUM OF VARIOUS ANIMALS

It seems that the substances produced by streptococci in artificial medium depends on the character of the medium. Thus Besredka [14] and Ruediger [15] demonstrated that the streptolysin produced in medium containing blood serum of different animals exhibits characteristic actions on the corpuscles of those animals. The experiments of Hellens indicate that there is produced different amounts of streptolysin according to the serum added to the broth. In order to ascertain whether there is any relationship between leukocidin production and different kinds of serum, I made experiments, the results of which are given in table 2, and as there shown the largest amount of production occurred in broth with goat or horse serum, than in that with rabbit serum, while that with guinea-pig serum gave the lowest amount.

TABLE 2

LEUKOCIDIN PRODUCTION IN VARIOUS KINDS OF SERUM 1 PART ADDED TO PLAIN BROTH 9 PARTS

Culture Filtrate	Exudate	Results			
		Goat	Guinea-Pig	Horse	Rabbit
1.0	0.5	+	+	+	+
0.75	0.5	+	+	+	+
0.5	0.5	+	0	+	+
0.25.	0.5	+	0	+	0
0.125	0.5	±	0	±	±
0.05	0.5	0	0	0	0
—	0.5	0	0	0	0
0.5 heated to 60 C. for 30 minutes	0.5	0	0	0	0

LEUKOCIDIN PRODUCTION AND TIME

According to Bail, the greatest amount of leukocidin is produced by staphylococci after a growth of 11 days, and Neisser and Wechsberg noted that production began in 4 days and it reached the maximum point after one week.

Substances produced by streptococci in cultures, such as acid and streptolysin, seem to reach the maximum at a fixed time and hence it

[14] Ann. de l'Inst. Pasteur, 1901, 15, p. 880.
[15] Jour. Am. Med. Assn., 1903, 41, p. 962.

would be reasonable to suppose that if leukocidal substances are formed by streptococci, they also will reach the maximum at a fixed time. The results in table 3 show that the greatest amount of leukocidin in this case was present after from 10-24 hours. This relationship is identical with the production of streptolysin. The amount of leukocidin falls after 48 hours and 72 hours of cultivation. The greatest amount of leukocidin appears in comparatively young cultures of streptococci.

TABLE 3

LEUKOCIDIN PRODUCTION TIME AND ACID FORMATION

Culture Fluid	Exudate	Age of Culture				
		5 Hours	8 Hours	15 Hours	24 Hours	48 Hours
0.75	0.5	+	+	+	+	0
0.5	0.5	±	+	+	+	0
0.25	0.5	0	+	+	0	0
0.1	0.5	0	+	+	0	0
0.05	0.5	0	0	0	0	0
0.5 heated to 60 C. for 30 minutes	0.5	0	0	0	0	0
Acidity of culture fluids	...	0.8	0.9	1.5	1.5	2.0

ACTION OF LEUKOCIDIN AND TIME

The action of leukocidin on leukocytes requires a little time. When I observed the degree of action in mixtures of leukocidin and leukocytes at different intervals I found that the action is as fully developed after 30 minutes as after the lapse of one hour, when it seems to remain stationary. It is also noteworthy that the rapidity of action depends on the amount of leukocidin that is added to the leukocytes; in the mixture in which a large amount of leukocidin is present the action is almost complete in from 30-60 minutes, while in mixtures containing less leukocidin, the same degree of action is realized only after from 1½-3 hours (table 4).

TABLE 4

LEUKOCIDIN ACTION AND TIME

Culture Filtrate	Exudate	Time and Results			
		½ Hour	1 Hour	1½ Hours	3 Hours
1.0	0.5	+	+	+	+
0.75	0.5	+	+	+	+
0.5	0.5	±	+	+	+
0.25	0.5	0	+	+	+
0.125	0.5	0	±	±	±
0.06	0.5	0	0	0	0
—	0.5	0	0	0	0
0.5 heated to 60 C. for 30 minutes	0.5	0	0	0	0

LEUKOCIDIN ACTION ON VARIOUS LEUKOCYTES

Are all leukocytes attacked alike by leukocidin or is there a difference in their susceptibility? In order to answer these questions I examined the action of streptococcus filtrate on human, rabbit, guinea-pig, rat, mouse and dog leukocytes, and I found very little difference in the result. The streptococcus used was the one obtained from a human abscess and passed through 5 rabbits and 15 mice.

As to the question whether the kinds of leukocytes are equally susceptible, I studied rabbit leukocytes. Polynuclear leukocytes were attacked first, while mononuclear leukocytes, particularly lymphocytes, showed strong resistance. In other words, even in case of contact with a large quantity of leukocidin for several hours, the lymph cells frequently remained unchanged. Thus it may be said that mononuclear leukocytes, particularly the lymph cells, are more resistant than other leukocytes.

According to my results, the susceptibility of the leukocytes in rabbits about one month old, is less than in older animals.

Neisser and Wechsberg found that staphyloleukocidin did not destroy kidney cells. I tested the action of streptoleukocidin on liver, kidney, spleen and marrow cells from rabbits killed by bleeding, the organs being cut into small pieces with a sterilized knife and the pieces put in four times their volume of 0.9% salt solution and agitated thoroughly. The cells thus freed were used for experiments, but I could not obtain any striking results. When the mixtures of cells and leukocidin were kept at 37 C. for from 3-4 hours, a comparatively large quantity of the leukocidin would kill the cells, especially liver cells; that is, 0.5-0.25 c c of leukocidin killed a certain number of cells within from 3-4 hours. Generally, the organ cells were much more resistant that leukocytes.

RESISTANCE OF LEUKOCIDIN TO HEAT, ETC.

The resistance of leukocidin to heat was examined with the results shown in table 5. There seems to be no effect after heating at from 45 to 48 C. for one hour; heating at 55 C. for one-half hour caused a little loss of action; when the leukocidin was left for one-half hour at from 58-60 C. it lost its power to attack leukocytes.

Investigation of the decrease or loss of leukocidal power under different conditions has shown that when kept in the icebox at 4-5 degrees, the leukocidin in streptococcus filtrates after 4 days suffered no change in power, but a decrease in power to 0.25 from 0.05 was noted after from 7-10 days. At 0 C. the leukocidin kept much better and its power showed no sign of change after 2 weeks. At 37 C. the power of the leukocidin did not decrease after 3, 7, and 15 hours, but it began to fall after 24 hours. At room temperature (about 26 C.), the leukocidin showed a marked decrease in power in from 2-4 days.

TABLE 5

EFFECT OF HEAT ON LEUKOCIDIN

Culture Filtrate	Exudate	Results		
		55 C. for 15 Minutes	55 C. for 30 Minutes	58 C. for 30 Minutes
1.0	0.6	+	+	0
0.75	0.6	+	+	0
0.5	0.6	+	±	0
0.25	0.6	+	±	0
0.125	0.6	+	0	0
0.05	0.6	±	0	0
0.025	0.6	0	0	0
—	0.6	0	0	0

Can leukocidin be reactivated? The results of many experiments to revive the activity of leukocidal streptococcus filtrates after being heated to 60 C. for 30 minutes, by addition of a small amount of active leukocidin were all negative. It looks as if streptoleukocidin once rendered inactive cannot be reactivated, and consequently in this respect it resembles the toxins.

DOES LEUKOCIDIN COMBINE WITH LEUKOCYTES AT LOW TEMPERATURE?

To 10 c c of leukocidal culture fluid diluted with an equal volume of 0.9% salt solution, an excess of leukocytes was added. The leukocytes used were obtained by centrifugating aleuronat exudate and suspending the cells in 0.9% salt solution. Such mixtures were left at room temperature or in the icebox for 3 hours, then centrifugated and the clear supernatant fluid examined according to the method described. It was found that the leukocidin and the leukocytes united, whether left at room temperature or in the icebox, as fresh leukocytes afterward were not attacked by the supernatant fluid.

SIMILARITIES AND DIFFERENCES OF STREPTOLEUKOCIDIN AND STREPTOLYSIN

Streptoleukocidin may be regarded as a toxin which affects leukocytes and streptolysin as a toxin which lakes red corpuscles, and it becomes necessary at this point to describe the similarities and differences of these two substances.

According to the results described previously,[11] streptolysin is most abundant in cultures about from 10-24 hours old. Only small amounts are found in cultures less than 5 hours or more than 48 hours old. With streptoleukocidin the condition is almost similar, the largest amount being obtained in the fluid of cultures between 10 and 24 hours old. Thus there seems to be no significant difference between leukocidin and streptolysin in point of time of production.

The power to produce streptolysin is retained by streptococci even when avirulent to animals and long cultivated artificially, but when streptococci are passed through animals, that is, become more virulent, the production of streptolysin is increased. Streptococcus leukocidin has a somewhat similar relationship, but I have found that nonvirulent streptococci may produce streptolysin freely but no demonstrable streptoleukocidin whatever. I have obtained lytic action by the culture fluids of several strains of streptococci when there was no trace of leukocidin, and it seems clear that a hemolytic streptococcus that produces no leukocidin nevertheless may give rise to streptolysin.

No hemolysis takes place in a mixture of streptolysin and red corpuscles kept at zero for from 3-5 hours, but the lysin and corpuscles combine, and the supernatant liquid obtained by centrifugating this mixture does not cause hemolysis. Similarly, in leukocidal experiments with the supernatant liquid no destruction of leukocytes occurs. When a mixture of leukocytes and leukocidin is kept at room temperature or in an icebox (4-5 C.) for 3 hours, a union of the two takes place, and the centrifugated fluid causes no leukocytic destruction, but this fluid is definitely hemolytic, its lytic power being much less, however, than that of the nontreated filtrate.

The resistance of streptolysin to heat is greater than that of streptoleukocidin, that is, leukocidin when heated at 55 C. for 30 minutes suffers but little effect. After being heated for half an hour at 58 C. it loses completely its power to attack leukocytes, while streptolysin when so heated retains its power to cause lysis.

ANTILEUKOCIDIC ACTION OF NORMAL AND ANTI-STREPTOCOCCUS SERUM

It has been found that the normal serum of different animals as a rule possesses some power to neutralize specific venomous substances. The action of normal serum on the leukocidin produced by streptococci has been studied, the serum being first heated at 56 C. for 30 minutes, to destroy any lytic action on rabbit leukocytes, and then a mixture of such heated normal serum and leukocidin kept at 37 C. for one hour when leukocytes were added. Generally speaking, the results indicated that the normal serum of different animals possesses antileukocidal properties and that human and horse serums possess this power in higher degree than certain other serums (see table 6). While heating normal serums for 30 minutes at 60-65 C. does not affect its antileukocidal power, heating at 70 C. for 30 minutes completely destroys this power. I have obtained results indicating that after recovery from scarlet fever the serum may have increased antileukocidal power.

TABLE 6

ACTION OF ANTILEUKOCIDAL RABBIT SERUM

Culture Filtrate	Exudate	Serum	Results			
			Normal Serum			Immune Rabbit Serum
			Human	Horse	Rabbit	
0.25	0.6	1.0	0	0	0	0
0.25	0.6	0.75	0	0	0	0
0.25	0.6	0.5	0	0	0	0
0.25	0.6	0.25	0	0	+	0
0.25	0.6	0.125	±	0	+	0
0.25	0.6	0.05	+	+	+	0
0.25	0.6	0.025	+	+	+	+
0.25	0.6	—	+	+	+	+

With a view to finding a solution to the question whether the antileukocidin in normal serum is derived from leukocytes by secretion, the effect of fluids in which leukocytes has been kept was tested, but the result was negative.

First, 50-100 c c of an equal volume of plain broth and 0.9% salt solution were injected intraperitoneally in a rabbit, and after from 6-8 hours the animal was bled to death and the exudate immediately removed from the peritoneal cavity. The exudate was mixed at once with salt solution, containing 1% sodium citrate and centrifugated, and the leukocytes separated and suspended in salt solution. The proper density of the suspension was found to be a mixture which in a 2 cm. test tube barely permitted letters to be seen through the fluid.

This suspension was left at 37 C. for 3 hours, and then again centrifugated. The upper clear liquid was tested for antileukocidal action.

To test whether leukocytic extracts are antileukocidal, leukocytes were obtained as described, and suspensions of the same concentration rapidly heated at 50 C. for 30 minutes, thus killing all the leukocytes contained therein. Then the suspension was left at 37 C. for 2 hours, centrifuged and the supernatant liquid tested for antileukocidal action; the results indicated clearly that the leukocytic extract had some antileukocidal action. Heated at 60-65 C. for 30 minutes such extracts were still antileukocidic, but when heated at 70 C. for 30 minutes this power was reduced markedly.

The question whether antistreptococcus serum has antileukocidal action is a significant one from the standpoint both of theory and practice. I have examined the action of antistreptococcus serum as follows:

A.—The strain of Str. pyogenes, isolated about one year ago from a human abscess, was used to immunize a rabbit. The first two injections were made with the bacteria heated at 60 C. from 24-hour cultures in 0.2% glucose serum broth and the last three with living bacteria. All injections were made intravenously. The blood was obtained for examination from 7-14 days after the last injection. The serum used agglutinated the streptococcus at 1:2,560 and had an opsonic index of 1.46.

B.—The streptococcus strain now used was a strongly virulent one, having been passed through 5 rabbits and 15 mice. The method of immunization was the same as just outlined. The serum in this case agglutinated at 1:1,280; while the coccus was nonphagocytable in normal rabbit serum, the immune serum caused marked phagocytosis. The results of the tests for antileukocidin were not decisive. Although rabbit B serum gave a stronger antileukocidal action than rabbit A serum and normal rabbit serum, the action was not so pronounced but that it may have been due to normal antileukocidin. And later, serum from a rabbit immunized with a streptococcus which had been passed through 5 rabbits and 24 mice was found to have no more antileukocidal action than normal rabbit serum. This immune serum agglutinated in 1:1,280; the streptococcus was nonphagocytable with normal rabbit serum, but with the immune serum an average of 8 cocci per leukocyte were taken up.

ANTISTREPTOLEUKOCIDAL SERUM

When a bacterial toxin is injected into the animal body, antitoxin may be produced. As streptococcus leukocidin seems to be a toxin produced by streptococci, the question arises whether a specific antileukocidin can be obtained. As the experiments just detailed indicate that we cannot demonstrate any antileukocidal action on the part of antistreptococcus serum, we might infer that immunization with leukocidin will not yield striking results.

Fullgrown and healthy rabbits were chosen for immunization. The material for injection was freshly made each time, and 8 injections were given at intervals of from 4-7 days. The amount for the first injection was 0.5 c c, and this amount was gradually increased to 2.0 c c. The first injection was given subcutaneously, the next intravenously, and alternatingly thereafter. The serum was obtained from 7-14 days after the last injection. The streptococcus strain used in this experiment had been passed through 5 rabbits and 27 mice and cultivated for 15 hours in a medium of plain broth and inactive goat serum 2-1.

According to the results of the tests (table 6), there seemed to be a distinct increase of anti-action of the immune as compared with normal serum, 0.05 c c of the immune serum having the same effect as 0.5 c c of normal serum. I have examined the antileukocidal action of normal rabbit serum many times, but I have not yet found such marked action as that of the serum of the rabbit immunized with leukocidin, and I am led to regard this action as due to the presence of an antibody. The antiserum had also a stronger neutralizing action on heterologous streptoleukocidin than normal serum.

Does antileukocidal serum promote phagocytosis? Virulent streptococci not only remain unmolested by leukocytes in phagocytic mixtures, but often the leukocytes are broken up and lost, due to the leukocidin produced by the cocci. Hence, when an antileukocidal serum that neutralizes leukocidin is added, the functional disturbances and destruction of leukocytes ought to be prevented. I have studied phagocytosis by guinea-pig leukocytes in mixtures of virulent streptococci (passed through 5 rabbits and 24 mice) and antileukocidal serum, and the average number of bacteria taken up by each leukocyte was 8, the largest individual number being 24. This streptococcus was absolutely nonphagocytable in normal serum. A heterologous streptococcus, nonphagocytable in normal serum, was rendered freely phagocytable by antileukocidal serum, the average number taken up per leukocyte

being 14. According to these results, antileukocidal serum not only accelerates the phagocytosis of the homologous streptococcus strain, but also facilitates the phagocytosis of heterologous strains.

In an experiment to test the protective power of this antileukocidal serum against streptococci, I used a 24-hour serum broth culture of which 0.001 c c killed mice. Of this culture 0.002 c c were injected intraperitoneally 15 hours after varying quantities of immune serum, heated at 56 C. for 30 minutes, had been injected subcutaneously. According to the results the immune serum had definite protective power.

COMPARISON OF STREPTOLEUKOCIDIN AND STAPHYLOLEUKOCIDIN

According to Neisser and Wechsberg's [10] results, the leukocidin produced by certain pyogenic staphylococci seems to be similar to the leukocidin produced by streptococci—the resistance to heat, the action on leukocytes, and the relation of production to virulence seem about the same. In my experiments Staph. aureus was used, and I found that 0.1 c c of the filtrate (Maasen filter) of a plain broth culture 4 days old killed leukocytes (filtrate 0.06 c c, exudate 0.5 c c, and salt solution 1.4 c c), but this effect was not suspended by the serum of a rabbit injected with streptoleukocidin, which, however, was neutralized by the serum (table 7).

TABLE 7

ACTION OF THE ANTISTREPTOLEUKOCIDAL SERUM AGAINST STAPHYLOCOCCUS LEUKOCIDIN

Serum (Normal or Immune)	Exudate	Antistreptoleukocidal Serum		Normal Serum	
		Strepto-leukocidin 0.2 c c	Staphylo-leukocidin 0.1 c c	Strepto-leukocidin 0.2 c c	Staphylo-leukocidin 0.1 c c
1.0	0.5	0	0	0	0
0.75	0.5	0	0	0	0
0.5	0.5	0	0	0	0
0.25	0.5	0	0	0	0
0.1	0.5	0	+	+	+
0.05	0.5	0	+	+	+
0.025	0.5	+	+	+	+
—	0.5	+	+	+	+
—	0.5	0	0	0	0

SUMMARY

Streptococci, like certain other bacteria, produce a toxic substance — apparently a toxin — that destroys leukocytes. In serum-broth medium the largest amount of streptoleukocidin is produced in from 10-24 hour; after that the production falls. The largest amount of

leukocidin was obtained in broth with goat serum and horse serum, the next largest amount with rabbit serum, while guinea-pig serum gave the least. There is a definite relation between the volume of production and virulence—virulent streptococci produce more leukocidin by far than avirulent, which may produce none at all.

Streptoleukocidin is rendered inactive when heated to from 58 to 60 C. for 30 minutes, and it is an unstable substance. Once rendered inactive it cannot be made active again by the addition of small quantities of fresh leukocidal culture fluid. The leukocidin and leukocytes unite at room temperature as well as in the icebox.

Normal serum and leukocytic extract possess antileukocidal action; this anti-action is lost when the fluids are heated at 70 C. for 30 minutes.

It seems difficult to produce antileukocidal effects by immunizing rabbits with streptococci, but easier by injecting leukocidal culture fluids. Antileukocidal immune serum not only exercises antileukocidal action against homologous and heterologous streptoleukocidin, but also possesses strong opsonic powers, rendering virulent streptococci, homologous as well as heterologous, easily phagocytable.

Streptoleukocidin appears to be distinct from streptolysin and antistreptoleukocidin does not neutralize staphyloleukocidin.

COMPLEMENT FIXATION IN TUBERCULOSIS, AND A COMPARISON OF THE WASSERMANN AND HECHT-WEINBERG-GRADWOHL SYSTEMS

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The lack of uniformity in results of fixation tests in tuberculosis obtained by different workers is due to several factors. Foremost, no doubt, is the wide divergence in the preparations used as antigens and in the quantities of the same antigen used by different workers. Practically all antigenic preparations made from the tubercle bacillus, if used in sufficient quantities, are anticomplementary, and some of the reports with 100% fixation, no doubt, were at fault in technic. Unless the quantity of hemolytic amboceptor is far in excess of that necessary in the Wassermann test, this false fixation will occur and be misleading.

Corper [1] pointed out that one difficulty, if not the main difficulty, in the complement fixation test for tuberculosis, is the close relation between the anticomplementary and the antigenic dose. I have observed that the time necessary for the primary incubation is longer when tuberculous serum and tubercle bacillus antigen are used than is the case with syphilitic serum and lipoid antigen. The failure to attach due importance to these facts may lead to error. In my earlier work I did not recognize, until after many tests had been made, that an entirely different technic must be employed wih tuberculous serum and tubercle bacillus antigen than with syphilitic serum with standard antigens.

The work now reported covers 635 tests on 570 patients. Fresh serum was used in each case.

The antigens used were prepared from different strains of tubercle bacilli, some of them isolated in this laboratory; other cultures were obtained from Dr. W. B. Wherry, University of Cincinnati; Dr. Lydia Dewitt, University of Chicago; Dr. E. R. Baldwin, Saranac Lake, N. Y.; Dr. J. B. Murphy, Rockefeller Institute; Dr. H. J. Corper, Municipal Sanatorium, Chicago, and from the American Museum of Natural History, N. Y. Other substances than the

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[1] Proc. Robt. Koch Society for the Study of Tuberculosis, 1916, 1, p. 105.

tubercle bacillus were used in parallel tests, e. g., salt solution suspensions of staphylococci, colon, typhoid, subtilis, smegma, lepra bacilli, Moeller's grass bacillus, glycerol broth boiled down to 1/10 its original volume and alcoholic extract of caseous glands. It was found that all preparations containing important constituents of the tubercle bacillus will act as antigens, but they also give a high anticomplementary titer.

Frazier[2] found that antigens from living, virulent tubercle bacilli had strong antigenic properties. Taking this for a working basis, antigens were made from living, virulent organisms according to the following formula: 50 mg. of tubercle bacilli were carefully weighed and placed in a sterile mortar and ground up with 90 mg. of sodium chlorid C. P. for one hour. To this 10 c.c. of sterile distilled water were added and the antigen was ready for use.

As a routine I used not more than ¼ of the anticomplementary dose of the antigen, 0.2 c c of patient serum inactivated for 15 minutes at 56° C., two units of guinea-pig complement and two and one-half units of hemolytic anti-sheep amboceptor titrated against fresh, washed sheep corpuscles. The primary incubation was one hour at 37° C. and the tests were read after 15 and 30 minutes' secondary incubation.

TABLE 1

Results Obtained with Tubercle Bacillus Antigens

Antigens	Classification											
	Far Advanced			Moderately Advanced			Doubtful			Negative		
	Serums Tested	% Positive	% Negative	Serums Tested	% Positive	% Negative	Serums Tested	% Positive	% Negative	Serums Tested	% Posi tive	% Negative
Miller's antigen	160	37.5	62.5	28	46	54	6	33	66	3	0	100
Petroff'sNaOH extract.......	150	50	50	18	45	55	5	20	80	4	0	100
Living, virulent tubercle bacilli........	135	70	30	46	61	39	9	33	66	9	66	33
Lepra..........	70	20	80	23	39	61	6	0	100	6	33	66
Smegma........	80	28	72	17	24	76	7	14	86	6	33	66
Bovine tubercle bacilli.....	53	30	70	17	53	47	6	33	66	3	100	0
Grass bacillus..	54	37	63	17	29	71	6	16	84	2	50	50
6 strains of tubercle bacilli	55	56	44	11	82	18	6	16	84	3	100	0
Methyl alcohol extract of B. tuberculosis..	61	70	30	13	70	30	3	0	100	6	0	0

The results obtained with the tubercle bacillus antigens (Table 1) show that in the far advanced cases, the greatest number of fixations was obtained with the antigen prepared with living, virulent tubercle bacilli and Petroff's methyl alcoholic extract,[3] both of them giving a 70% fixation. The sodium hydrate extract[3] gave better fixation than Miller's antigen.[4] In the moderately advanced cases the methyl alcoholic preparation gave the best fixation (70%); Miller's antigen gave 46% and the sodium hydrate extract 45% fixations.

[2] Cited by Corper (1).

[3] Am. Rev. Tuberculosis, 1917, 1, p. 33.

[4] Jour. Am. Med. Assn., 1916, 67, p. 1519.

Since practically all of the patients were in the moderately and far advanced stages, conclusions cannot be drawn from the results in the few doubtful and negative cases.

Antigens from living, virulent, bovine tubercle bacilli according to our formula gave a high percentage of fixation, 52% of 92 cases. Antigens from avirulent strains of tubercle bacilli showed a remarkably low percentage of fixation. Antigens from six strains of tubercle bacilli (3 human virulent bacilli, 2 virulent bovine and 1 avirulent), gave the highest percentage of fixation in the moderately advanced cases (82%).

In order to test the specificity of the reaction, antigens were made according to our formula from B. leprae, B. smegmatis and Moeller's grass bacillus. These antigens gave the following percentages of fixation: lepra, 25%, grass 37% and smegma 30%. With a suspension of staphylococci 24% of fixations were obtained and with B. coli 8%. The other antigens, such as glycerol broth and B. subtilis, gave an occasional fixation, while antigens from B. typhosus gave no fixation. The antigen from tuberculous caseous glands was found to have practically no antigenic value.

TABLE 2

RESULTS FROM THE WASSERMANN METHOD AND THE HECHT-WEINBERG-GRADWOHL SYSTEM

Antigens	Serums Tested	% Positive by the Wassermann System	% Positive by the H-W-G System
Frazier's	117	64	50
Smegma bacillus	54	24	11
Grass bacillus	39	33	33
Lepra bacillus	38	27	16
Alcoholic extract of beef heart	230	14.8	14.8

In table 2 are given the results with the Wassermann method and the Hecht-Weinberg-Gradwohl system.[5] It is seen at a glance that the results were less satisfactory with the Hecht-Weinberg-Gradwohl system when using the tubercle bacillus antigens; when using the alcoholic extract of beef heart, of 230 serums tested, 14.8% were positive with each system. A few serums gave positive fixations with the Wassermann system and not with the Hecht-Weinberg-Gradwohl system and vice versa; however, 98% of the serums gave the same fixation. Of 234 serums tested only 4 or 1.7% were unsuitable for the Hecht-Weinberg-Gradwohl method.

Of 620 patients, 118 or 18% gave positive Wassermann results. Of these 55% also gave positive complement fixation tests for tuberculosis, 96% being second and third stage tuberculous cases. Nine per cent. of the white patients and 34% of the colored gave positive Wassermann reactions. We feel that these figures are representative of the amount of syphilitic infection among the middle classes of the white and the general colored population of Cincinnati. A fair percentage of the whites giving a positive Wassermann reaction, deny a history of syphilitic infection and a history of such infection is a rare exception in a negro. There is no doubt that the Wassermann test is the best method we have for detecting syphilitic infections and the assumption that a positive Wassermann reaction is obtained in tuberculous patients without syphilitic infections has little support.

[5] Am. Jour. Syphilis, 1917, 1, p. 450.

DISCUSSION

A high percentage of tuberculous patients give a positive complement fixation with antigens prepared from the tubercle bacillus.

These antigens have a high anticomplementary titer and the difficulty existing is the close relation between the anticomplementary value and the antigenic dose. This fact is entirely overlooked by some observers. The 100% fixation in tuberculous cases obtained by Craig,[6] Miller and Castleman, Slack and Burns,[7] may have been due to this circumstance, and the small percentage of fixation obtained by Moursund [8] was probably due to the failure to recognize the lesser degree of fixation obtained with the tubercle bacillus antigens than with the syphilitic antigens.

Sufficient work is now recorded to establish the fact that a circulating antibody is present in the blood of a good percentage of tuberculous patients. There are, however, certain patients who do not show fixation with the antigens so far tested. It is highly probable that in some active cases fixation cannot be obtained.

It has been suggested that patients showing a high hemolytic index for sheep blood might give a negative reaction, and those giving a low index a positive reaction. By parallel tests with the Hecht-Weinberg-Gradwohl system, it was possible to study this point, and it was found that no such relations exist.

The sodium hydrate and methyl alcohol extractions of tubercle bacilli gave high percentages of fixations, but both are prone to become anticomplementary and the sodium hydrate preparation frequently has hemolytic properties. For all practical purposes the emulsions of living, virulent organisms give the best results. The reactions are clear-cut, either positive or negative, and if the suspensions are freshly prepared, they were not anticomplementary in quantities far in excess of that necessary to bind the complement and show no hemolytic properties. Contaminations soon render the antigen anticomplementary and new preparations should be made every six or seven days.

The results now reported with Miller's antigen were far less satisfactory than those obtained with virulent bovine tubercle bacilli. With the Miller antigen whole groups of serums reacted either positively or negatively, the results being due to the close relation existing between the anticomplementary and the antigenic dose.

[6] Jour. Am. Med. Assn., 1917, 68, p. 773.
[7] Jour. Am. Med. Assn., 1917, 68, p. 1386.
[8] Tuberculosis, 1919, 26, p. 85.

CONCLUSIONS

The complement fixation test in tuberculosis is a valuable aid when taken in conjunction with other means of diagnosis and treatment.

The reliability of this test has not been sufficiently established to be used as a criterion in the diagnosis or to determine the presence of activity in a known case of tuberculosis.

The phenomenon is not a specific antigen-antibody combination, but tends towards a group reaction. This fact has been substantiated by the recent work of Cooke.[9] Next to the tubercle bacillus, the other acid-fast organisms such as B. smegmatis, B. leprae and Moeller's grass bacillus, gave the higher degree of fixations. Other substances such as staphylococci, B. coli, B. subtilis and concentrated solutions of peptone gave occasional fixations.

Antigens from living, virulent tubercle bacilli seem to be the best preparations to use in routine tests.

The Hecht-Weinberg-Gradwohl system when used with tubercle bacillus antigens gave a lower degree of fixation than the Wassermann. When used with syphilitic serum the results were the same in 98% of the cases.

[9] Jour. Infect. Dis., 1919, 25, p. 493.

THE USE OF TISSUE IN BROTH IN THE PRODUCTION OF DIPHTHERIA TOXIN

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The production of diphtheria toxin regularly and of sufficiently high potency has long been a matter of primary importance. Although diphtheria toxin was among the first of the bacterial products to be discovered, but little is now known as to its composition or the basic substances from which it is produced. The well known uncertainty of toxin production and the seasonal variation in potency (MacConkey [1]) emphasize the importance of finding a method of producing toxin regularly and of satisfactory strength. A study of the literature on the nature of diphtheria toxin fails to supply much definite information.

An excellent résumé of the literature on the nature of this substance is contained in the paper by Robinson and Rettger.[2] It is of interest to mention a few of the conclusions of investigators having some bearing on the work which we are to report. It has been generally agreed that peptone was the important factor in enabling the diphtheria bacilli to produce toxin, Hida [3] believing that the secondary proteoses were the important constituents. Hitchens [4] and others found that simple peptone solution does not support the production of toxin. Dzierzgowski and Rekowski [5] believed that the toxin was a combination of certain bases and albumoses, as no toxin is obtained until the medium turns alkaline. The latter point was recently confirmed by Bunker.[6] The work of Hadley [7] on the production of toxin from protein-free mediums is open to criticism, since a whole culture and not a bacterial free filtrate was used for toxicity tests. Spronk [8] replaced meat infusion by an extract of brewer's yeast and claimed that he obtained stronger toxin and greater regularity in its production. Smith [9] found that the oxygen supply was an important factor and observed that 0.2% dextrose was favorable to toxin production on account of its acceleration of growth. No acid was formed from glycogen.

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[1] Jour. Hygiene, 1912, 12, p. 507.
[2] Jour. Med. Res., 1917, 36, p. 357.
[3] Ztschr. f. Hyg., 1908, 61, p. 273.
[4] Jour. Med. Res., 1905, 13, p. 523.
[5] Quoted from Robinson and Rettger.
[6] Jour. Bacteriol., 1919, 4, p. 379.
[7] Jour. Infect. Dis., 1907, Supplement 3, p. 95.
[8] Ann. de L'Inst. Pasteur, 1898, 12, p. 701.
[9] Jour. Exper. Med., 1899, 4, p. 373.

In view of the lack of Witte peptone, the former standard for toxin production, it seemed desirable to utilize only American peptone, especially since such a product would be constantly available. The proteose peptone manufactured by the Digestive Ferments Company and the peptone of the Parke-Davis Company have given uniformly good results with the method to be described. None of the other peptones tested gave as favorable results.

The medium used by us is essentially the "hormone" broth of Huntoon[10] and is prepared as follows: Finely ground lean veal is infused in twice its weight of water and is stored in the icebox for from 18 to 24 hours. It is then filtered through a wire gauze and heated to 80 degrees for 5 minutes. The infusion is thoroughly cooled and any fat present is removed from the surface. Two per cent. peptone and 0.5% sodium chlorid are added. The medium is brought to the boiling point and held there for 5 minutes. It is then titrated, using phenolphthalein as an indicator, to from neutral to 0.3% acid and again boiled 5 minutes. If the reaction is found to be correct the broth is filtered through wire gauze and the sediment allowed to settle. If necessary, the reaction is again corrected before filtration. The medium is distributed in 125 c c amounts in 300 c c. Florence flasks and sterilized 15 minutes at 15 pounds pressure. The reaction of the medium and its relation to toxin production is discussed in detail in another section.

In view of the fact that Bull and Pritchett[11] found that B. welchii produced more toxin in the presence of fresh sterile tissue, it was considered advisable to determine the effect on diphtheria toxin production of adding tissue to broth prepared in the manner already described. Various tissues from the rabbit and guinea-pig have been used, but guinea-pig liver tissue gives by far the best results. Liver tissue from guinea-pigs which have been negative to toxin tests are satisfactory, as no difference in results has been noted between tissue derived from these animals and that from normal pigs. The liver tissue was introduced into the flasks of broth in the following manner: A large guinea-pig was killed by a blow on the head or at the base of the skull and plunged into a 0.5% lysol solution for a few minutes. The body cavity was then asceptically opened and a mass of liver slightly larger than a 25-cent piece was introduced into each flask of 125 c c of medium. The liver tissue was not exsanguinated. Freshly drawn guinea-pig blood neither enhances nor inhibits the production of toxin. It seemed desirable to use guinea-pig tissue in order to avoid foreign protein toxicity in the diphtheria toxin tests. It has been found that when guinea-pig liver tissue is removed with care, according to the technic just described, it is nearly uniformly sterile for less than 1 out of 10 pigs appears to be infected. The liver tissue was transferred directly from the animal into the flasks of sterilized mediums and inoculated immediately with the culture of diphtheria bacillus because tissue which has stood in mediums in the incubator to test its sterility loses its power of enhancing toxin production. The reason for this loss of enriching property is not clear. Contaminations of the mediums from infected tissue or other sources can be detected in smear preparations at the time of testing the toxin and also by the odor of the broth.

[10] Jour. Infect. Dis., 1918, 23, p. 169.
[11] Jour. Exper. Med., 1917, 26, p. 119.

On account of the favorable results obtained by the addition of sterile liver tissue to broth, the effect of adding a water extract of beef liver tissue to broth in place of the tissue was tried but with negative results. Infusion broth prepared from beef liver instead of veal does not give toxin production comparable with the ordinary infusion broth to which guinea-pig liver has been added. Two explanations may be advanced for these results: (1) there may be some physical or mechanical reaction with the freshly excised liver tissue or (2) the essential substance is destroyed by sterilizing the liver infusion.

Inoculation of the tissue broth was made from fresh moist 24-hour cultures on Loeffler blood serum and luxuriant pellicle formation always resulted within 30 hours, provided the tissue was sterile. When the tissue was not sterile pellicle formation was not only greatly retarded, but the piece of tissue usually rose to the surface and floated, whereas sterile tissue invariably remained at the bottom of the flask. The Park-Williams strain No. 8 was used in all tests unless otherwise noted.

TABLE 1

The Effect of the Initial Reaction on Toxin Production

Initial Reaction	Incubation	Dilution of Toxin		Remarks
		0.01	0.005	
Neutral	4 days	Not tested	Pig died after 3 days	Heavy pellicle
Plus 0.1	4 days	Pig died after 2 days	Pig died after 2 days	Heavy pellicle in 36 hours
Plus 0.3	4 days	Not tested	Pig died after 2 days	Good pellicle
Plus 0.45	6 days	Pig died after 6 days	Pig died after 7 days	Pellicle formed slowly
Plus 0.5	5 days	Negative	Negative	Reaction in 0.01 pig
Plus 0.5	7 days	Pig died after 3 days	Negative	Pellicle formed in 48 hours
Plus 0.8	8 days	Negative	Not tested	Pellicle formed in 36 hours
Plus 1.0	8 days	Negative	Not tested	Poor pellicle formation
Neutral (control)	6 days	Negative	Negative	0.1 toxin produced

Since the purpose of this study was to find a method that would assure the regular production of toxin of usable strength rather than one which would supply an occasional high titer toxin interspersed with many nontoxin producing starts, a minimum lethal dose of 0.005 was set as a standard. Tests of considerably higher dilutions of tissue enriched broth were occasionally made and the results in most cases were positive. While not all the toxins produced were tested at a strength of 0.003 there is every evidence to show that most of them were at least of that titer while several had a titer greater than 0.0025. Broth prepared with Parke-Davis peptone and not enriched with liver tissue usually produced a low potency toxin with occasional toxins of a titer up to 0.005. Proteose peptone without tissue produced a 0.005 toxin or one of somewhat higher strength with greater regularity but in our hands 25% of the lots of medium prepared with this peptone failed to produce a 0.005 toxin.

As Bunker[8] has pointed out, the initial reaction of the broth determines to a considerable extent the potency of the toxin produced, as well as the character and speed of pellicle formation. Table 1 gives the initial reactions of different lots of broth varying from neutral to 1% acid to phenolphthalein.

Parke-Davis peptone was used in the preparation of each lot shown in this table. Tissue was used in the broth in each case unless otherwise indicated.

The reactions to phenolphthalein were checked by hydrogen-ion readings by the colorometric method and mediums showing reactions to phenolphthalein ranging from neutral to plus 0.3% fall within or near the limits indicated by Bunker.[6] It seems worthy of notice that equally satisfactory results are obtainable when the reaction of diphtheria toxin broth is adjusted by either the phenolphthalein or hydrogen-ion method. Table 1 indicates that in broth to which tissue has been added the most favorable initial reaction range to phenolphthalein is from neutral to plus 0.3%. As the reaction approaches plus 1.0%, toxin production becomes more and more irregular and finally ceases entirely. When toxin is formed in mediums with acid reactions of more than plus 0.3% to phenolphthalein the titer is lower than in mediums prepared at the same time and from the same veal infusion but with a reaction below plus 0.4%.

The initial reaction also appears to bear a fairly direct relation to the rate and luxuriance of pellicle formation for with mediums ranging from neutral to plus 0.3% a heavy, firm pellicle is formed within 24 hours and increases in thickness for about 3 days, after which it appears to remain constant. With a higher concentration of acid, pellicle formation is greatly retarded and the pellicles when finally formed are frequently incomplete or extremely thin and easily destroyed by slight agitation of the medium. The same conditions regarding the effect of the initial reaction of the medium on toxin production and pellicle formation seem to apply to proteose peptone as table 2 demonstrates.

TABLE 2

THE EFFECT OF THE INITIAL REACTION ON TOXIN PRODUCTION

Initial Reaction	Incubation	Dilution of Toxin		Remarks
		0.01	0.005	
Neutral	4 days	Pig died after 3 days	Pig died after 4 days	Heavy pellicle
Plus 0.2	4 days	Pig died after 2 days	Pig died after 5 days	Heavy pellicle
Plus 0.5	7 days	Negative	Negative	Slow pellicle formation
Plus 0.8	7 days	Not tested	Pig died after 8 days	Slow pellicle formation
Neutral (control)	4 days	Pig died after 4 days	Negative	Good pellicle formation
Neutral (control)	5 days	Not tested	Pig died after 2 days	Heavy pellicle

In testing the toxins shown in tables 1 and 2 due account was taken of the fact brought out by Bunker[6] that when the initial reaction is acid a longer incubation period must be allowed for the reversal to the alkaline reaction. It appears, however, that even when incubation is prolonged toxin is of low potency if the initial reaction varies to any great extent from the optimum.

Although the initial reaction of broth is of the greatest importance, it is equally necessary that the reaction become markedly alkaline if a high titer toxin is to be produced. Bunker[6] has already pointed out this fact and experiments with broth enriched with liver tissue confirm his observations. A tissue enriched flask seldom shows a reaction more alkaline than control flasks to which no tissue has been added. In the great majority of cases the reactions of the control flasks increase in alkalinity at a more rapid rate after the third

day or sometimes the fourth day of incubation. This point is discussed later. Table 3 shows the initial reaction, the toxin production and the reaction at the time of testing of a lot of broth prepared with Parke-Davis peptone.

The interesting fact is brought out by this table that although the final hydrogen-ion reaction may be in a range where toxin is produced, nevertheless, if the initial reaction is too alkaline there is no toxin production. Here again the period of incubation was taken into account in order that sufficient time might be allowed for toxin production. When the reaction before testing has reached a value of P_H 8.0 one may expect to find a potent toxin in so far as this detail is concerned. It appears evident that toxin is only produced in broth which has a final reaction of at least P_H 8.0 and that stronger toxins usually have a more alkaline reaction up to P_H 8.3. A favorable reaction at the time of testing is not, however, an indication that toxin has been produced, for many lots of mediums without tissue enrichment had hydrogen-ion reactions within ranges where high titer toxins would be expected but with negative tests in guinea-pigs. It is evident that while the initial and final reaction of the medium has a direct bearing on toxin production other factors must also be favorable if the medium is to yield toxin.

TABLE 3

The Effect of Initial and Final Reaction on Toxin Production

Initial Reaction		Toxin Production		Reaction before Filtration, P_H
Phenolphthalein	P_H	0.01	0.005	
—0.1	7.9	Negative	Negative	8.0
Neutral	7.6	Died after 4 days	Died after 4 days	8.0
Neutral	7.6	Died after 4 days	Died after 4 days	8.0
Neutral	7.7	Not tested	Died after 3 days	8.2
Plus 0.4	7.3	Died after 3 days	Died after 3 days	8.3
Plus 0.4	7.2	Not tested	Died after 3 days	8.3
Neutral (control)	7.3	Died after 11 days	Negative	8.2
Neutral (control)	7.4	Died after 2 days	Died after 3 days	8.1

The addition of sterile liver tissue to broth appears to reduce the necessary period of incubation considerably. Most investigators have reported that incubation of not less than 6 days and usually longer is required. According to Bunker[6] this period is closely bound up with the progressive change in the reaction of the broth into an alkaline range. While this may be true, the constituents of the broth and particularly those substances that reduce the lag period and greatly enhance rapid growth, shorten the necessary incubation period for the production of a favorable alkaline reaction. When tissue is added to broth the optimum range of alkalinity appears to extend over a longer period of time than in unenriched mediums. Apparently the liver tissue has a buffer action independent of the peptone but this point has not been determined absolutely.

Diptheria bacilli grow with much greater rapidity in broth to which a piece of sterile liver tissue has been added than in unenriched mediums. Pellicle formation is far more luxuriant and the pellicle shows as a white film in from 18 to 24 hours, while a pellicle of equal strength and thickness is not formed on normal broth until from 30 to 36 hours have elapsed. The increase in strength and thickness of the pellicle after it is formed is more rapid when tissue has been added and at any time during incubation the enriched broth presents a more favorable pellicle for toxin production than control flasks of the same lot of medium. It is natural to expect that when

growth and luxuriant pellicle formation are so rapid less time would be required for the production of a toxin of reasonably high potency. Table 4 gives the toxin production over a series of days of flasks filled with the same lot of broth and inoculated under as nearly the same conditions as possible. Parke-Davis peptone was used in the preparation of this broth and the reaction was neutral to phenolphthalein.

TABLE 4

RELATION OF THE PERIOD OF INCUBATION TO TOXIN PRODUCTION

Period of Incubation	Dilution of Toxin	
	0.01	0.005
4 days	Not tested	Died after 4 days
4 days (without tissue)	Negative	Negative
5 days	Not tested	Died after 5 days
5 days (without tissue)	Negative	Negative
6 days	Not tested	Died after 3 days
6 days (without tissue)	Negative	Negative
7 days	Not tested	Died after 2 days
8 days	Died after 2 days	Died after 3 days
8 days (without tissue)	Died after 2 days	Died after 3 days

TABLE 5

RELATION OF INITIAL REACTION, RATE AND POTENCY OF TOXIN PRODUCTION AND FINAL REACTION

Period of Incubation	Dilution of Toxin		Final Reaction
	0.01	0.005	
3 days—tissue	Not tested	Died after 2 days	P_H 8.0
3 days—control	Not tested	Negative	P_H 7.9
4 days—tissue	Not tested	Died after 4 days	P_H 8.1
4 days—control	Not tested	Negative	P_H 8.1
5 days—tissue	Not tested	Died after 2 days	P_H 8.1
5 days—control	Died after 2 days	Died after 2 days	P_H 8.2
6 days—tissue	Not tested	Died after 3 days	P_H 8.2
6 days—control	Not tested	Negative	P_H 8.3
7 days—tissue	Not tested	Negative	P_H 8.3
7 days—control	Not tested	Negative	P_H 8.4

This series of tests was selected because one of the flasks of broth without tissue enrichment produced a 0.005 toxin and this fact allows a comparison with tissue enriched mediums in relation to speed of toxin production. Unfortunately the final reaction of this series of tests was not recorded, but similar series in which the P_H value was taken showed that broth enriched with sterile liver tissue attains a favorable alkaline reaction usually on the third day provided the initial reaction ranged from neutral to plus 0.3% to phenolphthalein as shown in table 5. The control flasks filtered on the fourth, fifth and sixth days did not contain a 0.01 toxin but on the eighth day a 0.005 toxin had been produced. It is evident from this table that broth without tissue enrichment requires longer incubation than flasks filled with the same lot of medium to which sterile pieces of liver tissue have been added. Table 5 brings out the relation between the initial reaction the rate and potency of toxin production and the final reaction. In this lot of broth proteose peptone was used and the initial reaction was neutral to phenolphthalein or P_H 7.7. A flask with tissue and a control were inoculated for each of the 5 days and tested as indicated.

This table presents clearly the advantage derived from adding tissue to broth which produces toxin. When the initial reaction is in the correct range for toxin production tissue enriched broth reaches a favorable alkalinity usually on the third or fourth day and the reaction continues advantageous over a considerable period of time. Furthermore, this table confirms the results recorded in table 4, for toxin production was more rapid in broth containing tissue than in the same lot of medium unenriched. When tissue is added to broth there appears to be less danger of collecting the toxin at an incorrect period of incubation since toxin is frequently found in tissue free broth on only one or two days during the whole course of the experiment.

The effect of an initial acid reaction on the rate and potency of toxin production is brought out in table 6. In this lot of broth proteose peptone was used and the initial reaction was plus 0.4 to phenolphthalein or P_H 7.2. A flask with tissue and a control were inoculated for each the 5 days.

TABLE 6

THE EFFECT OF AN UNFAVORABLE ACID REACTION ON TOXIN PRODUCTION

Period of Incubation	Dilution of Toxin		Final Reaction
	0.01	0.005	
4 days—tissue	Negative	Negative	P_H 8.0
4 days—control	Negative	Negative	P_H 7.9
5 days—tissue	Died after 2 days	Negative	P_H 8.2
5 days—control	Negative	Negative	P_H 8.1
6 days—tissue	Not tested	Died after 6 days	P_H 8.3
6 days—control	Negative	Negative	P_H 8.3
7 days—tissue	Died after 2 days	Died after 3 days	P_H 8.3
7 days—control	Negative	Negative	P_H 8.4
8 days—tissue	Not tested	Died after 7 days	P_H 8.4
8 days—control	Not tested	Died after 6 days	P_H 8.3

The same point is again brought out in this table as in table 3. The fact that the final reaction has reached a favorable degree of alkalinity is no guarantee that toxin has been produced. The effect of liver tissue on broth of poor toxin producing qualities is well shown in table 6. While the control flask of the eighth day of incubation contained a 0.005 toxin of poor quality, the flask with tissue enrichment on the seventh day showed a good quality, 0.005 toxin and one of the same strength but poorer quality on the sixth day. This table again confirms the results recorded in table 4, since toxin was produced with greater rapidity in broth containing sterile tissue.

Since the purpose of this study was to devise a method of producing diphtheria toxin regularly, little attention has been given to the exact potency of the product provided it was at least 0.005. Some of the control lots of broth, particularly that prepared with proteose peptone, produced toxins of a strength as high as 0.0033 and even 0.0025. In such cases the corresponding flask of broth with tissue added gave an equally high titer and within a shorter period of time. Occasionally a broth with tissue enrichment was tested at 0.0033, and in most cases was positive, although the control flask frequently did not contain a 0.01 toxin. Tests have been made on 18 lots of tissue enriched broth prepared with proteose or Parke-Davis peptone, the initial reaction ranging from neutral to plus 0.3% to phenolphthalein. In each of

these lots a 0.005 toxin was obtained from flasks incubated from 3-7 days, the optimum period of incubation appearing to be about 5 days. Broth has been prepared by other methods than that previously described, but with unsatisfactory results. Flasks of mediums in which tissue was incubated 24 hours to test for sterility before inoculation with the culture of B. diphtheriae failed to produce toxin. Evidently, the liver tissue must be absolutely fresh to be of value in enhancing toxin production. Similar results were obtained by Bull and Pritchett [11] in their study of the toxin production of B. welchii.

Several strains of B. diphtheriae have been tested for toxin production by this method and encouraging results have been obtained. These cultures were isolated from active or convalescent cases, and had been cultivated on Loeffler blood serum slants for from 1-12 months. They had never been grown in broth up to one week before the tests were made when they were planted in tubes of "Hormone" broth to which small pieces of liver tissue had been added to encourage pellicle formation. After transferring a few times, pieces of the pellicles were transplanted onto the surface of broth in flasks after the tissue had been added. Eleven strains of B. diphtheriae were tested in this manner, of which but one failed to produce toxin. Eight of the remainder produced a 0.02 toxin, one had a strength of 0.006, while one killed in a dilution of 0.004. These tests still further support the belief that the addition of liver tissue to broth is of great value in the production of toxin.

The explanation of the effect of tissue on toxin production is extremely difficult owing to the lack of knowledge of the chemistry of tissue, especially in relation to the metabolism of bacteria. Certain theories may be advanced, however. Liver tissue, owing to its high glycogen content, furnishes an easily available food for the bacteria. Since B. diphtheriae does not form acid from glycogen no effect on the reaction of the medium results, even though it is rapidly attacked. Again, the tissue may absorb some of the oxygen in solution in the broth, thereby causing the diphtheria organisms to grow in thick pellicle formation on the surface in order to obtain an adequate supply of oxygen. The fact that only fresh tissue is of value indicates that this theory may be at least a partial explanation of the favorable results, for tissue which has stood for twenty-four hours in uninoculated broth has probably reached its limit of oxygen absorption. The surface of such a medium, unprotected by pellicle formation, is still in direct contact with the oxygen of the air which the broth probably continues to

absorb. Finally, liver tissue may supply in extra quantities substances similar to those found by Spronk [8] in yeast cells, which he claimed to be essential to toxin production. Since glandular tissue is particularly rich in vitamins, the presence of these substances in liver tissue may have an important effect on toxin production. Perhaps no one of these theories completely explains the favorable action of liver tissue, but rather a combination of them. The presence of both glycogen and accessory substances in the tissue seems to us, however, to account for the rapid growth and regular toxin production in broth which has a favorable reaction. In a study of the hemotoxin production of streptococci we have found that there is in fresh tissue some substance of great delicacy essential for the elaboration of hemotoxin. In many respects there appears to be a similarity between the substances from which diphtheria toxin and the hemotoxin of streptococci are derived. The presence of glycogen and accessory substances in tissue seems to us, however, to be presumptive evidence that they play an important part in the rapid and regular toxin production.

No toxin has been produced by this method on a commercial scale owing to a lack of proper facilities. It is therefore impossible to state whether or not the trouble and expense of adding liver tissue to broth would be warranted under commercial conditions when large amounts of toxin must be produced. These observations, however, may be of value in suggesting new lines of attack on the question of the constitution of diphtheria toxin.

CONCLUSIONS

Diphtheria toxin can be produced regularly in broth to which pieces of sterile guinea-pig liver have been added. The medium must be inoculated immediately after the addition of the tissue.

Broth prepared with certain American peptones gives satisfactory results when enriched with liver tissue.

The most favorable initial reaction of the broth ranges from plus 0.3% to neutral to phenolphthalein.

The broth at the time of testing should have a reaction ranging from P_H 8.0 to P_H 8.3, although a favorable reaction is not the only essential for toxin production.

The addition of liver tissue reduces the necessary period of incubation.

Strains of B. diphtheriae other than Park-Williams No. 8 have produced a toxin of high potency by this method.

THE INFLUENCE OF CREOSOTE, GUAIACOL AND RELATED SUBSTANCES ON THE TUBERCLE BACILLUS AND ON EXPERIMENTAL TUBERCULOSIS

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XIX.

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Of the countless drugs used in tuberculosis, creosote and its derivatives undoubtedly hold first place in extent of use and general reputation, but absolutely without scientifically established value. Perhaps as good a summarization of their status as any is given by Lawrason Brown,[1] who says that creosote and its derivatives "are the most used of false specifics. They have never been proved to exert any action on the tuberculous process but in some patients have almost a specific action upon the accompanying secondary infection of the lungs, such as simple bronchitis. They also exert a very stimulating effect upon the bronchial mucous membrane during their excretion through it." Other authors vary somewhat in their judgment, but positive statements are avoided, of necessity. Kobert says, "Very little that is conclusive can be said concerning the usefulness of these preparations." Bandelier Ropke's "Die Klinik der Tuberkulose" contains the conclusion that "Creosote and guaiacol preparations are not internal disinfectants, but for certain cases they stimulate the appetite and improve digestion. Their routine use is therefore by no means justifiable."

A careful examination of the extensive literature on the many compounds of this class indicates that opinions as to their value or action, whether favorable or unfavorable, rest upon very slender evidence. General clinical experience is the only extensive source of information, and this is, of course, uncontrolled and conflicting, so it

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1. Klebs: Tuberculosis, New York, D. Appleton & Co., 1909.

does not serve as a basis for conclusions of any value whatever. The most that can be said is that the use of creosote therapy in tuberculosis has persisted so long and so widely that it seems probable that some beneficial results must have been observed. Medical history gives us the right to suspect that any methods or substances of therapeutics that continue to be generally used for many years have some definite favorable effect, although the real establishment of their principles and limitations may not be understood. Sometimes after years of clinical use, and sometimes even after long subsequent years of disuse, the explanation of their activity has been established.

There has been no lack of endeavor by chemical manufacturers to provide all possible derivatives of creosote and its components, as can be seen in any list of chemical products, but especially in Fraenkel's "Arzneimittelsynthese." These endeavors have, with few exceptions, not been based on considerations of bactericidal efficiency, and indeed this essential aspect has been almost completely neglected. As Fraenkel says in summarizing this section of his book, two objects underlie the preparation of these multitudinous derivatives of creosote and guaiacol—(a) reduction of the toxicity and irritating properties, together with improvement in taste; (b) water solubility. Most of the products accomplishing the first objects, largely by means of the salol principle, are not water-soluble. The water-soluble compounds are mostly bad tasting, and, in case of the sulfonates, are much reduced in activity. "In therapeutic application, in the first rank of products indisputably come those formed by esterification of the hydroxyl group." He also comments that of the active components of creosote only the guaiacol has had general use in pure form, while the less toxic cresols, which have analogous effects, have had no consideration.

There are numerous compilations of literature on creosote therapy in tuberculosis (under which term are included the numerous derivatives of creosote and related substances), and we shall not give another compilation. One of the best reviews is that by von Weismayr in Ott's "Chemische Pathologie der Tuberculose." We give, however, in brief form such scanty evidence as we have found concerning the bactericidal action of this class of compounds, both in vitro and in vivo:

Bouchard is quoted by Weismayr as having found that 0.8 per 1,000 strength of creosote in glycerol bouillon retards the growth of tubercle bacilli, and 0.5 per 1,000 is effective in blood serum. In daily doses of 0.25 gm. per kilo it caused immunity to tuberculosis in rabbits, so that animals killed after three months showed no lesions. We regret that we have not been able to find the origin of this statement, for it is perhaps the most definite one in the literature.

On the other hand, Cornet[2] reported that he inoculated 7 guinea-pigs with tuberculosis after they had been given 0.02 gm. creosote daily per catheter into the stomach for about a month, and continued after infection, but they all died of tuberculosis as did the control animals. This dose he estimated as corresponding to 2.0 gm. creosote per day in a man. He also quotes Schüller as obtaining positive results and Sormani and Pallacani as obtaining negative results by causing infected guinea-pigs to inhale creosote.

Guttmann[3] studied the inhibition of growth of different species of bacteria on gelatin containing creosote, which he found to be more effective than phenol. Of 18 species of bacteria, 12 failed to grow when the gelatin contained one part in 2,000 (including 4 not growing at 1 : 4,000), and 5 of the remaining 6 failed to grow when the concentration was 1 : 1,000. With 17 species on gelatin containing phenol, 12 grew when the concentration was 1 : 2,000. Tubercle bacilli were partly inhibited by creosote 1 : 4,000 and even 1 : 16,000, but it required 1 : 2,000 to prevent growth entirely. He then speculated as to the possibility of obtaining a concentration of 1 : 2,000 of creosote in the human body, and found that this could not be done, wherefore he was doubtful as to the efficiency of creosote in tuberculosis—a speculation that has been quoted extensively in literature on tuberculosis.

Fraenkel[4] corroborated in 1889 the statement that cresols are stronger antiseptics than phenol. He quotes von Behring's estimate that one-sixth the amount of an antiseptic found to be inhibiting for bacteria represents approximately the lethal dose for an animal. In accordance therewith, Fraenkel found that 1 : 300 cresol sulfate is inhibiting to bacteria (species not stated) which would correspond on this basis to two-sixths gm. in a 600 gm. pig, an amount found fatal although a slightly smaller dose was not fatal.

Kuprianow[5] quotes Petrescu as finding that tubercle bacilli did not grow in 10 c c bouillon containing 4.1 gm. (sic) guaiacol, and that tubercle bacilli exposed to guaiacol did not produce tuberculosis. Marfori also found that guaiacol rendered tubercle bacilli unable to infect rabbits. No other references to direct observations on the bactericidal power of guaiacol could be found in the literature to 1894 by Kuprianow. He therefore made some tests by Loeffler's method, in which the solution to be tested is poured over the surface of an inoculated agar slant, and poured off again after a specified time, the tube then being incubated. In other experiments 24 to 48 hour slant cultures were exposed to the solutions and material removed with a platinum loop was inoculated on fresh mediums. The inhibiting effect in bouillon cultures was also determined. Phenol and creosote were found by these methods to be about equally destructive to staphylococci and B. pyocyaneus, but guaiacol was less active than either. To all 3 the staphylococcus was much more resistant than the pyocyaneus bacilli. Tubercle bacilli were exposed in masses of culture to 4% alcoholic solution of guaiacol and creosote for 15 seconds to 2 hours, and after pouring off the solution no growth followed. Obviously these last experiments are too crude to be of any significance.

Koch is quoted as having found that creosote inhibits the growth of tubercle bacilli in cultures, but the reference cited in the literature is incorrect, and the original article has not been located.

[2] Ztschr. f. Hyg. u. Infektionskr., 1888, 5, p. 124.
[3] Ztschr. klin. Med. 1888, 13, p. 488.
[4] Ztschr. f. Hyg. u. Infektionskr.. 1889, 6, p. 521.
[5] Centralbl. f. Bakteriol., 1894, 15, p. 933.

Winkler[6] exposed agar plate cultures of tubercle bacilli to vapor from a mixture of guaiacol and iodoform for eight days and found that the material became noninfectious. Injection of this mixture into animals did not save them from tuberculosis, and serum of rabbits injected with the antiseptic had no effect on infection with tubercle bacilli.

Villa is quoted by von Weismayr as having found that guaiacol prevents growth of streptococci in a dilution of 1 : 1,000, and kills in this dilution in 16 minutes, and in dilution of 1 : 100 in 2 minutes.

Hammerl[7] found that paracresol is equal to orthocresol in bactericidal power against staphylococci and typhoid bacilli, but more toxic. Phenol was less strongly bactericidal and more toxic than either.

Several authors quote Shaw[8] as having demonstrated that guaiacol is ineffective in infections of animals, but the original article shows that he merely inoculated two rabbits with B. pyocyaneus, and injected one with 20 c c of a 1 : 200 guaiacol solution (the lethal dose of which is 25 c c). This animal died in 18 hours and the control in 26 hours. There is no other experimental evidence in this much quoted article.

One of the most important contributions to the subject of creosote therapy is that of Bechhold and Ehrlich,[9] who (using especially diphtheria bacilli for their tests) developed many new and fundamental facts in relation to the influence of various modifications of the phenol derivatives on their bactericidal and physiologic action. The chief conclusions were:

1. Introduction of halogens into phenol increases the disinfectant action in proportion to the number of halogen atoms introduced[10] (e. g., 1 mol. pentabrom phenol has the same action on diphtheria bacilli as 500 mol. phenol).

2. Alkyl groups introduced into phenols or halogen phenols increases their disinfectant action. (Tribrom-m-xylenol is 20 times as active as tribrom phenol; tetrabrom-o-cresol is 16 times as active as tetrachlor phenol).

3. Union of 2 phenols or halogen phenols, either directly or through CH_2, CHOH, $CHOCH_3$ or $CHOC_2H_5$ groups, increases activity. Thus, tetrabrom-o-cresol inhibits the growth of diphtheria bacilli in a dilution of 1 : 200,000, while tetrabrom-o-biphenol inhibits when dluted to 1 : 640,000.

4. Union of 2 phenols through CO or SO_2 decreases activity.

5. Introduction of COOH into the nucleus decreases activity.

6. Halogens introduced into phenols at first reduce toxicity, but the trihalogens have about the same toxicity as the unhalogenized substance, and tetra- and penta-halogen compounds are more toxic. However, the spasmodic action of the phenols is reduced in proportion to the number of halogen atoms. CH_3 groups compensate or neutralize the toxicity introduced by the halogens.

Of the compounds developed in this study the most effective were:

Tetrabrom-o-cresol, which has but little toxicity yet inhibits growth of diphtheria bacilli diluted to 1 : 200,000, and in 1% solution kills them in less than 2 minutes. It compares in activity with phenol in the ratio of 1,000 : 0.9

Tetrabrom-o-biphenol (and the corresponding Cl compounds) which is more toxic but inhibits growth at a dilution of 1 : 640,000.

Hexabrom-diphenyl carbinol, practically nontoxic, inhibits growth at 1 : 200,000; kills in 24 hours in dilution of 1 : 320,000 and kills in 10-15 minutes at

[5] Deut. med. Wchnschr., 1893, 19, p. 781.

[6] Hyg. Rundschau, 1899, 9, p. 1017.

[8] Jour. of Hygiene, 1903, 3, p. 159.

[9] Ztschr. physiol. Chem., 1906, 47, p. 173.

[10] In a Patentschrift, Dammann, in 1889, also mentions this effect of halogens; quoted by Schottelius, Arch. f. Hyg. 1913, 82, p. 76.

1 : 1,000. Compares with phenol as 1,000 to 0.6 in respect to action on diphtheria bacilli, although less effective against "water bacteria" than phenol. Although these substances did not precipitate proteins they were ineffective against diphtheria bacilli in serum, and on this basis the authors explain their failure to influence favorably diphtheria infection in animals Unfortunately, they give no details as to the methods used in conducting these experiments. They merely say "Wir versuchten Hexabromdioxydiphenyl carbinol, Tetrabromhydrochinonphthalein usw. besonders gegen Diphtherie an Meerschweinchen, Kaninchen, und auch gegen Streptococcen an weissen Mäusen, Tetrabrom-o-Kresol gegen Streptococcen an weissen Mäusen. Der Erfolg blieb aus."

This article was followed by a report by Bechhold[11] under the title of "Halbspezifische chemische Desinfektionsmittel" in which is emphasized the fact that the effect of a given chemical on one species of bacteria may not be duplicated with another species, and hence general laws covering the influence of various modifications of a substance on its bactericidal action cannot be deduced from limited observations. Thus, in the previous article it was stated that the introduction of halogen atoms into phenols increases the disinfectant action somewhat in proportion to the number of added halogens. But Bechhold finds that against staphylococci, streptococci and diphtheria bacilli the maximum disinfectant power is shown by tribrom-β-naphthol, as compared with either mono- and di- or tetra- and penta-brom-β-naphthol. On the other hand, against paratyphoid bacilli the activity is constant as halogens are added to dibrom or dichlor, and decreases with three or more halogen atoms. The "semi-specificity" of this class of disinfectants is shown by several examples. Thus, tetrabrom-p-biphenol and tribrombikresol are very actice disinfectants for staphylococci, but against colon bacilli they are less effective than lysol. While tri- or tetrabrom-β-naphthol, tetrabrom-o-cresol and tetrachlor-l-biphenol have a considerable disinfectant action even on anthrax spores, they as well as some others of the higher halogen phenols, are practically inactive against tubercle bacilli. Tetrabrom-o-cresol, hexabromdioxydiphenyl-carbinol, tetrachlor-o-biphenol, tetrabrom-biphenol and tribromcresol, in 1% solution for 2 hours with an emulsion of human tubercle bacilli did not impair their infectivity for animals. Tri- tetrabrom-β-naphthol acted in 2.5% solution on tubercle bacilli for 25 hours without effect, while a 5% lysol solution (containing 2.5% of cresol) kills tubercle bacilli in 4½-8 hours. Hence all these disinfectants which are much more actively destructive of staphylococci than lysol, are much less effective than lysol against tubercle bacilli.

As far as we can learn, the leads given in these articles have not been followed much farther, either in Ehrlich's laboratory or elsewhere. Leubenheimer[12] has established anew the general applicability of the principle of the effect of halogenized phenols to bacteria, and also demonstrated for different xylenes a high bactericidal action (Schottelius).

Raschig[13] is said to have followed this lead and produced a chlorinated xylenol which has great bactericidal properties.

Schottelius[14] has investigated the action of "grotan," described as "a complex cresol alkali compound," Na-p-chlor-m-cresol. This substance he found to be strongly bactericidal, 0.5% solution killing in 5 minutes all the bacteria

[11] Ztschr. f. Hygiene u. Infektionskr., 1909, 64, p. 113.

[12] Phenol und seine Derivate, Berlin, 1909; quoted by Schottelius.

[13] An incorrect reference to Raschig's work is given and we have not succeeded in locating the correct reference.

[14] München. med. Wchnschr., 1912, 59, p. 2674.

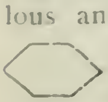
tried (typhoid stools and cultures, staphylococci, streptococci and pus), 0.3% usually being effective, and 0.25% killing in 20-30 minutes; 1% solution killed anthrax spores in 20 minutes. Tuberculous sputum was treated with an equal volume of 2% grotan for 10, 30 and 60 minutes and each injected into guinea-pigs and rabbits without infection resulting, although all the controls were tuberculous after 28 days. No further or more exact tests seem to have been made with tubercle bacilli. The substance is said to be almost nontoxic and nonirritating, 3 gm. subcutaneously not poisoning 4,000 to 6,000 gm. dogs.

He also reports[15] that in a mixture of chlorxylenol and chlorcresol the disinfectant action is not merely the sum of the components, but is increased about 100% above this. Especially efficient is a preparation made by dissolving chlorxylenol in soap and adding to "grotan." A new compound called "sagrotan" is produced in this way, but no exact statement is made as to its preparation or composition, beyond announcing its production by Schülke and Mayr. This substance he found much more strongly bactericidal for anthrax spores, staphylococci, streptococci, typhoid bacilli, and pus or dejecta containing these organisms, than either lysol or liquor cresoli saponatus. Also tuberculous sputum was disinfected by 2% sagrotan in 2 hours, as shown by animal inoculation. It is almost entirely devoid of either local or systemic toxicity. Dogs took 10 gm. per kilo (1% of body weight) into the stomach without serious effects, and Schottelius took 15 gm. at one dose and held his arm 40 minutes in a 10% solution of sagrotan without serious effects. However, 100 gm. in 3 days to a 7.5 kilo dog did not remove intestinal bacteria.

Sagrotan was found by Friedenthal[16] to have an alkalinity, as sold in 10% solution, corresponding to 0.56% KOH, so that injected subcutaneously it causes local necrosis. Guinea-pigs are not affected by subcutaneous injection of 2.5 gm. per kilo. In general, the low toxicity of grotan and sagrotan was corroborated by Friedenthal, who does not discuss their bactericidal effects.

Fehrs,[17] in discussing various preparations of liquor cresoli saponatus, mentions that Staph. pyogenes aureus is very susceptible, and Streptococcus pyogenes intermediate.

An extensive consideration of the effect of electrolytes in disinfection with cresol soaps is given by Frei,[18] but this contains nothing bearing directly on our problems.

Heukeshoven[19] has made the most extensive study of the action of thiocol, and one of the few studies of the effect of creosote derivatives on tuberculous animals that we can find in the literature. He found that a-thiocol,  OCH_3 OH SO_3K and b-thiocol, SO_3K  OCH_3 OH were almost absolutely without inhibiting effect on staphylococci, anthrax and B. pyocyaneus, for all these grew in asparagin glucose agar containing from 1 to 5% of these compounds; while guaiacol carbonate, being used merely as a suspension, had little more effect, but K-guaiacolate prevented growth of staphylococci in 0.5% (the lowest concentration tried), and inhibited anthrax and pyocyaneus at 2% but not at 1%.

The animal experiments were performed with four series of rabbits inoculated with tubercle bacilli (origin not stated) in the eye. In each series were

[15] Arch. f. Hyg., 1914, 82, p. 76.

[16] Berl. kl. Wchnschr., 1915, 39, p. 1019.

[17] Centralbl. f. Bakteriol., 1, 1904, 37, p. 730.

[18] Ztschr. Infektkr. d. Haustiere, 1914, 15, p. 273 and 407.

[19] Experimentelles über die Wirkung des "Thiocols" bei der Tuberkulose, R. Heukeshoven, Inaug. Dissert., Bern, 1899.

nine rabbits, one serving as a control. They received daily doses of 0.5 gm. of each of the 4 guaiacol derivatives mentioned above, by means of a catheter. In one series the drug was given for 14 days before infection and not afterward; in the second the treatment was continued; in the third there was no preliminary treatment, but treatment continued after the infection; in the fourth treatment was not begun until 4 weeks after the inoculation. The result was, in brief, that in the rabbits receiving thiocol-a, 2 recovered completely and in none was the disease disseminated through the body; with thiocol-b, none recovered, in 6 there was no dissemination, and in 2 there was dissemination; with potassium guaiacolate, in 5 of 8 the tuberculosis was disseminated; with guaiacol carbonate it was disseminated in 6 of 8, and in the 4 controls. He also found increase of weight in animals given thiocol-a or guaiacol carbonate, potassium guaiacolate had a deleterious effect on the animals and thiocol-b had no effect. As it has been found that thiocol is excreted unchanged, and as it seems to be devoid of bactericidal properties (although not tested on tubercle bacilli) these favorable effects are difficult to explain.[20]

Lubowski[21] has reviewed the abundant literature on thiocol to the middle of 1904, but this literature contains no work that seems to be accurate except that of Heukeskoven, nor have we found any contributions of importance since that date.

Diethylglycine-guaiacol, $C_6H_4 \begin{cases} OCH_3 \\ O\text{-}CO\text{-}\ CH_2\text{-}N\text{-}(C_2H_5)_2\ HCl \end{cases}$

known commercially under the name Guiasanol, has been produced and described by Einhorn[22] as having many therapeutic advantages. Gastric irritation is avoided because the free base is not formed until the HCl is split off in the intestinal alkalies, and free guaiacol is eventually liberated since it is found in the urine.[23] The toxicity is low, rabbits being uninjured by 2 gm. subcutaneously, or 4 gm. by stomach, and 2% solutions are not irritating. Buchner tested the bactericidal action of guiasanol, finding it low in vitro, for it inhibited the growth of B. coli, B. pyocyaneus, B. proteus and S. pyogenes aureus only in concentrations of 1:50 or 1:100. If it is correct that guaisanol readily liberates guaicol in the body, the low bactericidal power in vitro is of little significance, and Einhorn claims that it is efficient as a local antiseptic in ulcerating cancer and tuberculous enteritis.

A few other incidental references complete the list.

Burow[24] reported that a 3% aqueous solution of sodium or potassium guaiacolate containing 0.01% potassium arsenite prevented growth of tubercle bacilli, although without the arsenic the guaiacol was ineffective. He also stated that the blood of a rabbit treated with this mixture would not support growth of tubercle bacilli, and that guinea-pigs and rabbits thus treated resisted tuberculous infection. These results he attributed chiefly to the arsenic. This

[20] Lynosol, which differs from Thiocol in having Ca in place of K, is said by Takenaka (Japanese Jour. Tuberc. Kekkakau Zassi, 1919, 2, No. 1) to be as strongly bactericidal for tubercle bacilli as creosote and guaiacol, which all, he says, inhibit growth at concentrations over 1:10,000.

[21] Allg. Med. Centr.-Ztg., 1905, 74, pp. 337, 356, 396.

[22] München. med. Wchnschr., 1900, 47, p. 10.

[23] Concerning absorption and elimination of guaiacol see Eschele, Ztschr. klin. Med., 1896, 29, p. 197.

[24] München. med. Wchnschr., 1910, 57, p. 1792.

[25] München. med. Wchnschr., 1911, 58, p. 2669.

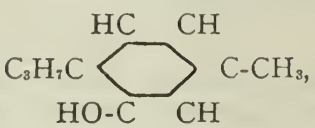
work, the report of which does not give a convincing impression, was repeated by Nürnberger,[25] who found no effects produced in either cultures or animals by the guaiacol-arsenic mixture of Burow, or by 0.01 gm. Na guaiacolate in glycerin-agar, the concentration not being stated.

J. Naberly[26] reported favorable clinical effects with a "new guaiacol chloriodid compound," the exact nature of which is not given. As there is no experimental evidence in regard to this compound, it is mentioned only because it has some possible relation to Ehrlich's observations. The Lancet laboratory examined this compound and found the iodin and chlorin nearly all in chemical union.

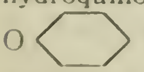
Cooper[27] reports an extensive study of creosote and allied substances, particularly with reference to the disinfectant action of various soap solutions in surgery and disinfection work. The chief facts developed of interest in this connection are:

Using the Rideal-Walker method as modified by Chick and Martin, the phenol coefficient of the cresols in pure aqueous solution was found to be:

	B. typhosus	S. pyogenes aureus
Ortho cresol	2.6	2.1
Meta cresol	2.6	2.0
Para cresol	2.6	2.4
Thymol	25.0	...
"Cresylic acid"	...	2.2

Therefore, as shown by thymol, 

, alkyl groups in the benzene ring may increase greatly the germicidal action, which accords with Bechhold's and Ehrlich's statements, and with the observation made by Koch in 1881.

On the other hand, the introduction of a second OH group into phenol decreases bactericidal action. Thus, for typhoid bacilli in water the phenol coefficient of various OH compounds was: resorcin, 0.3; pyrocatechin, 0.5; hydroquinone, 1.0; pyrogallol, 0.77; phloroglucin, less than 0.35. Quinone, 

, however, had a phenol coefficient for staphylococci of 10, whereas acetone was less than 0.075.

Krauss,[28] in describing several new compounds of trypan red, gives the method of making guaiacol trypan red and iodo-guaiacol trypan red. He says nothing concerning their activity, but Paul Lewis in a brief note published elsewhere[29] states that no effects had been obtained in experimental tuberculosis with any of their trypan red compounds.

BACTERICIDAL AND BACTERIOSTATIC EXPERIMENTS

In order to determine whether there is any reason for believing that the various members of the guaiacol series should be expected to have any direct action on tuberculosis, the inhibitory or bacteriostatic action

[26] Lancet, 1913, 2, p. 285.
[27] Brit. Med. Jour., 1912, 1, p. 1234.
[28] Jour. Amer. Chem. Soc., 1914, 36, p. 960.
[29] Jour. Pharm. and Exper. Therap., 1914, 4, p. 353.

of a considerable number of the series has been tested on the bacillus of human tuberculosis. Several strains of the organism were used; in some cases no note as to the strain was made. In most of the tests, a strain which had been growing in our laboratory for a number of years and which we have distinguished from other strains acquired more recently, by the name "old human" was used. This strain has diminished somewhat in virulence but has not changed its growth characteristics. We use the following method for testing inhibitory power: To tubes containing a certain definite amount of glycerol agar, the chemical to be tested is added in sufficient quantity to make the desired dilutions. The dilutions range from 10% up to 0.0001%. Two of the substances used (styracol and guaiacol cacodylate) are quite insoluble in water. To make dilutions of these, the required amount of dry powder was added to hot agar, well shaken, and the agar cooled and slanted quickly before the powder settled out. Table 1 gives a summary of the experiments on the inhibitory action of all the tested drugs of this series.[30]

From this table, it may be seen that 0.01% or 1 in 10,000 dilution completely inhibited the growth of the tubercle bacillus in the test with resorcin, thymol, p-cresol, m-cresol and o-cresol; that 0.05% was the lowest concentration which completely inhibited in the case of creosol and pyrocatechin; guaiacol, creosote, hydroquinone and guaiacol cacodylate required a concentration of 0.1% or 1 in 1,000 to inhibit growth completely. Sodium guaiacolate inhibited completely at 1.7% and partially at 0.8%. Thiocol and styracol caused no inhibition at 1% concentration while the organisms grew well even in a suspension of 10% of styracol, which seems to be almost completely insoluble in water.

The bactericidal power of many of these compounds was then tested in the following way. The "old human" strain was used in all the tests. Six dilutions of the chemicals to be tested, from 1 in 100 to 1 in 1,000,000, were made in water. Small clumps of cultures were then immersed in these solutions, remaining in them 10 minutes, 1 hour, 6 hours, 24 hours and 48 hours. At the end of the desired time, the clump was removed, washed in several waters to remove the chemical and finally planted in agar tubes. Controls were made by exposing the clumps to normal salt solution for the same periods of time and then washing them in the same way. They all grew luxuriantly.

[30] Some of these experiments were carried out by Dr. Rachel Donnell.

The tubes inoculated with the clumps which had been exposed to 1 per cent. orthocresol for one hour developed a slight growth, while the 6 hour, 24 and 48 hour sets showed no growth. One per cent. metacresol, paracresol and thymol killed all the cultures after one hour's exposure, while 1 per cent. thymol killed all the organisms in 10 minutes. There was but little growth in the tubes inoculated with the clumps exposed for 10 minutes to 1 per cent. metacresol and paracresol. Creosol, resorcin, hydroquinone and pyrocatechin had no bactericidal effect in this experiment even in 1 per cent. concentration for 48 hours

TABLE 1

Inhibition of Growth of Human Tubercle Bacilli

Percentage	Creosote			Guaiacol			O-cresol			M-cresol			P-cresol			Thymol			Creosol			Resorcin		
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-
0.8																								
0.5										—	—	—	—	—	—				—	—	—			
0.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	-
0.05							—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
0.01	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	++	++	++	—	—	-
0.001	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+++	+++	+++	+	+	[illegible]
0.0001	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+++	+++	+++	+	+	[illegible]

Blank spaces indicate that that dilution was not used.
The — sign shows that there was no growth (complete inhibition).

and concentrations below 1 per cent. had no bactericidal effect with any of the drugs, even 0.1 per cent. thymol failing to kill in 48 hours, although 1 per cent. killed completely in 10 minutes.

Creosote and guaiacol were also tested by the same method but different dilutions were used. One hundred per cent. of either creosote or guaiacol killed all the organisms in 5 minutes so that there was no growth on the agar tubes. Five per cent. dilution killed some in 5 minutes and killed all in 1 hour; 1% creosote killed all in 1 hour and in 6 hours, but it required 6 hours for complete tuberculocidal action of 1% dilution of guaiacol. There was no growth in tubes after one hour's exposure to 0.5% dilution of creosote and 6 hours and 24 hours exposure to 0.1% creosote killed some of the organisms. Guaiacol, on the other hand, showed little bactericidal action in concentrations lower than 1%.

From these results, it may be seen that the bactericidal power of these substances is much lower than the bacteriostatic power. One per cent. was the only concentration which had any marked bactericidal power as shown by this method; 1% thymol killed all the organisms even in 10 minutes, so that they failed to grow when planted on agar tubes; 1% of metacresol and paracresol killed in 1 hour and of orthocresol in 6 hours. Even 1% of creosol, resorcin, hydroquinone or pyrocatechin failed to kill. One per cent. creosote killed K_4 bacillus in one hour and the Y. Miller strain was killed in one hour by 0.5% of creo-

TABLE 1—*Continued*

INHIBITION OF GROWTH OF HUMAN TUBERCLE BACILLI

Thiocol			Styracol			Sodium Guaiacolate			Guaiacol Cacodylate			Hydro-quinone			Pyro-catechin		
+++	+++	+++	+++	+++	+++				—	—	—	—	—	—	—	—	—
						— +	— +	— +									
+++	+++	+++	+++	+++	+++				—	—	—	—	—	—	—	—	—
+++	+++	+++				+	+	+	—	—	—	—	—	—	—	—	—
+++	+++	+++	+++	+++	+++				+	+	—	++	++	++	—	—	—
+++	+++	+++	+++	+++	+++	+	+	+	+++	+++	+++	+++	+++	+++	+	+	+
+++	+++	+++	+++	+++	+++	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++

The + sign shows growth, ++ more luxuriant growth and +++ very luxuriant growth. Controls were grown in all cases and always showed very luxuriant growth.

sote. It required 5% guaiacol to kill the "old human" in 1 hour although 1% killed all the organisms in 6 hours.

We realized that the clump and test tube method of bactericidal work could not be considered absolutely reliable, since some of the clumps may not be completely permeated by the chemical while others will be saturated and broken up so that they are difficult to transplant to new mediums. For these and other reasons, the results as given above are not entirely consistent. Therefore it seemed best to repeat the bactericidal experiments with some, at least, of the compounds, using more delicate methods for determining the results. We chose the so-called garnet method, which we used thus:

1. A sufficient number of garnets of fairly uniform size to allow ten for each animal, were thoroughly cleaned with sulphuric acid, water, acetic acid, water, alcohol and finally ether. They were well

dried and sterilized. A suspension of tubercle bacilli was then made in which the garnets were well shaken and allowed to stand for a time. The fluid was decanted off and the garnets dried over sterile calcium chlorid. When dry, the required number of garnets were placed on perforated platinum baskets and immersed in about 50 c c of the various dilutions for the desired periods of time. The dilutions used were 1%, 0.5%, 0.1% and 0.01% and the periods of exposure were 20 minutes, 1 hour, 6 hours and 24 hours. Controls were exposed to physiological sodium chlorid solution for the same periods of

TABLE 2

Animal Test of Bactericidal Action

Dilutions	Duration of Exposure	Creosote						Guaiacol					
		I		II		III		I		II		III	
		Local	General	Local	General	Local	General	Local	General	Local	General	Local	General
1%	20 minutes	+	—	—	—	+	—	—	—	+	—	+	—
	1 hour	—	—	—	—	—	—	+	—	+	—	+	—
	6 hours	+	—	+	+	—	—	+	—	—	—	—	—
	24 hours	—	—	—	—	—	—	+	—	+	—	+	—
0.5%	20 minutes	+	—	+	—	—	—	+	—	+	—	+	—
	1 hour	+	—	—	—	—	—	+	—	—	—	—	—
	6 hours	—	—	—	—	—	—	+	—	—	—	+	—
	24 hours	—	—	+	—	—	+	—	—	+	—	—	—
0.1%	20 minutes	+	+	+	+	+	—	+	+	+	+	—	—
	1 hour	+	+	+	+	+	—	+	+	+	+	—	+
	6 hours	+	+	+	+	+	+	+	+	+	+	+	+
	24 hours	+	+	+	+	—	+	+	+	+	+	+	+
0.01%	20 minutes	+	+	+	+	+	—	+	+	+	—	+	+
	1 hour	+	+	+	+	+	+	+	+	+	+	+	+
	6 hours	+	+	+	+	+	+	+	+	+	+	+	+
	24 hours	+	+	+	+	+	+	+	+	—	+	+	+

Controls were inoculated with cultures which had been exposed to physiologic salt solution for the same periods of time and otherwise treated in the same way as the medicated cultures. All the control animals exhibited tuberculous lesions, either local or general or both.

time and then treated in the same way. At the end of the exposure, the basket with the garnets was lifted out and washed through three dishes of water and one of physiological salt solution. Then the garnets were transferred into sterile test tubes, each containing 2 c c of sterile physiological sodium chlorid solution, 10 garnets being counted into each tube and 3 or 4 tubes being allowed for each set. These tubes were then shaken thoroughly for one-half hour in a shaking machine to remove the bacteria from the garnets, and the salt solution was then injected subcutaneously into guinea-pigs, the entire amount in one tube being used for one pig. Therefore, there were 3 guinea-pigs in each set, or 60 for each experiment. In this method, the bacteria are in a

thin layer on the garnets so that there is no question of permeation of the layer. There is much less danger of contamination from handling and the guinea-pig response to inoculation is much more delicate than that of the agar tube. Table 2 gives a summary of the results of these experiments.

From this table, it may be seen that 1% was bactericidal in all the compounds except resorcin and even resorcin had in that concentration some bactericidal power, especially if the exposure lasted 24 hours. Creosote and guaiacol had marked bactericidal power in 0.5% concentration and thymol killed practically all the organisms in 0.1% dilution.

TABLE 2—*Continued*

ANIMAL TEST OF BACTERICIDAL ACTION

Orthocresol						Thymol						Resorcin					
I		II		III		I		II		III		I		II		III	
Local	General	Local	General	Local	General	Local	General	Local	General	Local	General	Local	General	Local	General	Local	General
—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—	+	—
—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—
—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	+	+
—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—
+	+	+	+	+	+	—	—	—	—	—	—	+	+	+	+	+	+
—	—	+	+	+	+	—	—	+	+	—	—	+	+	+	—	+	—
+	+	+	+	+	+	—	—	—	—	—	—	+	+	—		+	+
—	—	+	+	+	+	—	—	—	—	—	—	+	+	+	+	+	+
+	+	+	+	+	+	—	—	—	+	—	—	+	—	+	+	+	+
—	+	+	+	+	+	—	—	—	—	—	—	+	+	+	+	+	+
+	+	+	+			—	—	—	—	—	—	+	—	+	+	+	+
+	+			+	+	—	—	+	—	—	—	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	—	—	+	+	+	+	+	—	+	+
+	+	+	+	+	+			+	+	+	+	+	+	—	—	—	—
+	+	+	+	+	+	—	—	+	+	+	+	+	+	+	+	+	—

The + sign in the tables indicates that the bacteria developed either in the test tube or in the animal body. If, in table 2, the development of the tuberculous process occurred only in the lymph glands near the point of inoculation, the + sign is under "local." If other glands or other organs in the body are involved, the sign is placed in the column marked "general." It may be noted that results are not entirely consistent even by this method and that the — sign occasionally occurs where we have every reason to expect the + sign, even in the controls. This is probably due to the fact that in the shaking machine some of the tubes may be so placed that the garnets are not shaken violently enough to release a sufficient number of bacteria to cause an infection.

It may in some cases be due to the fact that some of the garnets are not well dried so that the bacteria are washed off before ready for shaking and hence are lost in the germicide or in the wash waters. The results are, however, sufficiently consistent to give us a definite idea of the limit of bactericidal action.

Therapeutic Experiments

Creosote.—Tests on toxicity of creosote were made in order to determine a safe dosage:

One guinea-pig received an intracardiac injection of 3 c c of $\frac{1}{200}$ creosote in physiological salt solution or 0.015 c c creosote with no ill effects.

One guinea-pig received an intraperitoneal injection of the same amount, 0.015 c c, with no ill effects.

TABLE 3

Therapeutic Test of Creosote (Set 1)

Duration of Treatment	Total Amount Creosote Received	Local Glands	Liver	Spleen	Lungs	Internal Glands
90 days	Fed 0.462 c c Injected 0.067 ½ c c	—	—	—	—	—
204 days	Fed 1.05 c c Injected 0.067 ½ c c .	—	+ ?	—	—	—
293 days	Fed 1.506 c c Injected 0.067 ½ c c	—	—	—	—	—
280 days	Fed 1.44 c c Injected 0.067 c c	+++	+++	—	—	++++
180 days	Fed 0.924 c c Injected 0.067 c c	++	+++	+++	+++	++
112 days	Fed 0.546 c c Injected 0.067 c c	+++	+++	+++	—	+++
141 days	Fed 0.726 c c Injected 0.067 c c	+++	+++	++++	—	+++

One guinea-pig received a subcutaneous injection of 0.1 c c creosote in physiological salt solution with no ill effects and no ulceration of the skin.

Series 1.—These 3 pigs and 6 normal pigs were then inoculated with 0.2 c c dilute emulsion of "old human" tuberculosis.[31] These 9 pigs were fed daily, except Sunday, one pill containing 0.003 c c creosote. Each received in addition one intramuscular injection of 0.005 c c creosote in 1 c c cotton seed oil, one intracardiac injection of 0.01 c c creosote in 2 c c physiological salt solution, 5 subcutaneous injections of 0.005 c c in 1 c c physiological salt solution and 3 subcutaneous injections of 0.0075 c c each in 1 c c physiological salt solution. The injections were made once a week for 10 weeks and the feeding was continued until death. Two of the pigs died immediately after the intracardiac injection, too early to show much general involvement, although the inguinal glands were enlarged and caseous. Table 3 gives results in the other 7 animals.

[31] In all these experiments, the animals of series 1 were inoculated with an amount of culture determined, not by weighing but by the opacity of the suspension. Sufficient sodium chlorid solution was added to make the suspension slightly opalescent. In the animals of series 2, the amount was determined by weight.

Series 2.—In this series, 9 guinea-pigs were inoculated subcutaneously with 0.2 mg. of Corper's 1305[32] in 0.2 c c of physiological salt solution. These were fed daily 0.2 drop creosote in 2 drops cotton seed oil. Five subcutaneous injections, one each week, were given of 2 drops creosote in 1 c c physiological salt solution.

Table 4 gives the results.

TABLE 4

THERAPEUTIC TEST OF CREOSOTE (SET 2)

Duration of Treatment	Total Amount Creosote Received	Local Glands	Liver	Spleen	Lungs	Internal Glands
84 days	Fed 14.22 drops Injected 9.0 drops	+++	+++	+++	+++	+++
105 days	Fed 18.0 drops Injected 9.0 drops	+++	+	+++	+++	+++
123 days	Fed 21.2 drops Injected 9.0 drops	+++	+++	+++	+++	+++
126 days	Fed 21.6 drops Injected 9.0 drops	+++	+++	+++	+++	+++
126 days	Fed 21.6 drops Injected 9.0 drops	+++	+++	+++	+++	++++
128 days	Fed 22.0 drops Injected 9.0 drops	+++	+++	+++	+++	+++
133 days	Fed 22.8 drops Injected 9.0 drops	+++	+++	+++	+++	+
152 days	Fed 26.0 drops Injected 9.0 drops	+++	+++	+++	+++	+++
154 days	Fed 26.4 drops Injected 9.0 drops	+++	+++	+++	+++	+++
220 days	Fed 37.8 drops Injected 9.0 drops	+	+++	+++	+++	+

Four of this set had definite and some fairly large cavities in the lungs—an unusual proportion.

Table 5 gives for comparison the conditions in untreated control guinea-pigs inoculated with the same dose of the same two strains of tubercle bacilli. It may be noted that some of the controls of set 1 failed to develop the disease even after 334 days. In other words, the treatment seems, with creosote, as well as with many other drugs, to lower the resistance of the animal to the disease.

TABLE 5

CONTROLS OF SET 1 OF THIS SERIES

Duration of Treatment	Local Glands	Liver	Spleen	Lungs	Internal Glands
64 days	+	—	—	—	+
96 days	—	—	—	—	—
97 days	+	—	—	—	+
287 days	+	++	+++	+++	+
334 days	—	—	—	—	—
461 days	—	+	++	+	—

Guaiacol Treatment Experiments.—Dec. 15, 1916, to determine the safe dose of guaiacol, 3 guinea-pigs were injected with guaiacol, 1 : 200 of physiological salt solution. One received 3 c c by intracardiac injection, one 3 c c by intra-

[32] This culture was isolated by Dr. Corper from the sputum of a tuberculous patient at the Chicago Municipal Tuberculosis Sanatorium about 1915, and has been grown in this laboratory ever since; 0.05 mg. injected subcutaneously into guinea-pigs, causes in every case generalized tuberculosis with death in from 4 to 6 months.

peritoneal injection and one received 2 c c by subcutaneous injection. As there were no ill effects except slight infiltration and induration of tissue surrounding the point of subcutaneous injection, the 3 were inoculated with 0.2 c c of a dilute suspension of "old human" tubercle bacilli and 6 other pigs received the same inoculation. Then each of the 9 received daily by mouth a pill containing 0.003 c c guaiacol and also an injection weekly. One injection was intramuscular, one was intracardiac, and seven were subcutaneous. One of these pigs died after 20 days, showing only involvement of a regional lymph gland, while 1 lived 929 days and exhibited at death no tuberculous involvement either local or general. Only 5 of the 9 guinea-pigs in this series showed advanced general tuberculosis; this corresponded fairly well with the controls of the same series in which only 2 of the 6 infected animals showed a general tuberculosis.

Guaiacol Treatment.—Set 2. In this set, Corper's 1305 was used and 0.2 mg. was injected subcutaneously. Ten guinea-pigs were inoculated at the same time. They were fed daily at first 0.2 drop in 2 drops cotton seed oil, then 0.3 drop and later 0.5 drop. Weekly subcutaneous injections of guaiacol were given in physiological salt solution, 1 drop at first, quickly increased to 2 drops in 1 c c of physiological salt solution. Of this set, one died in 21 days, having no tuberculous involvement. All the others exhibited a marked general tuberculosis of lymph glands, liver, spleen and lungs, the degree of involvement being about the same as that shown in the control animals of series 2, summarized in table 6. The last one of the set to die lived 191 days and had received a total of 90 drops of guaiacol.

TABLE 6

Controls for Set 2 of all these Experiments

Duration of Disease	Local Glands	Liver	Spleen	Lungs	Internal Glands
84 days	+++	++	++	+	—
98 days	+++	+++	+++	+++	+++
107 days	++	+	+	+	++
107 days	+++	+++	+++	+++	+++
114 days	+++	+++	+++	+++	+++
122 days	+++	++++	++++	+++	+++
138 days	+++	++++	++++	++++	+++
159 days	+++	+++	++++	++++	+++
163 days	+++	++++	+++	+++	+++
223 days	+++	++++	+++	+++	+++

Two of the animals had lungs containing cavitiees.

Creosol Treatment Experiments.—The first set consisted of 9 guinea-pigs, 3 of which had been previously used to determine the safe dose of creosol, and all were inoculated in January, 1917, with 0.2 c c of dilute emulsion of "old human." Four died or were killed too early to show any development of the disease. Two of these died immediately after an intracardiac injection, one from pneumonia and one from being crushed in the cage. In 2 of these early deaths, in which the guinea-pigs had lived 2 weeks, the animals showed early signs of tuberculosis, such as enlarged glands, and one had a few young tubercles in the lungs. The others were fed daily 0.2, 0.3 and 0.5 drop of creosol in cotton seed oil up to the time of death, and received 11 subcutaneous injections of creosol in physi-

ological salt solution at weekly intervals. Four of the 5 remaining animals in this set showed generalized tuberculosis, a much larger proportion than was seen in the untreated controls of the same set.

Creosol Treatment.—Set 2 was inoculated Feb. 4, 1918, with 0.2 mg. of culture 1305 and fed daily 0.2 of a drop for 12 days, 0.3 of a drop, then the rest of life 0.5 of a drop in cotton seed oily daily, except Sundays. The animals were injected weekly for 6 weeks with 2 drops in physiological salt solution. Ten guinea-pigs were used in this series. The first to die lived 80 days and the last 212 days; all showed a marked generalized tuberculosis, corresponding to the untreated controls of the same series (see table 6).

Thiocol Treatment.—Series 1: Jan. 3, 1917, six pigs were inoculated subcutaneously with 0.2 c c weak suspension of "old human" culture. From Jan. 4 on, they were fed daily one 5 mg. pill. After Jan. 18, they were fed two 5 mg. thiocol pills daily. On Jan. 10 and each week thereafter they were injected subcutaneously 5 mg. in 1 c c physiological salt solution the first 3 weeks and afterward 10 mg. in 1 c c physiological salt solution. The thiocol was easily soluble and seemed to have no ill effects. Four of the 6 animals of this set showed marked generalized tuberculosis.

Thiocol Treatment.—Series 2: Ten guinea-pigs were inoculated Feb. 4, 1918, subcutaneously with 0.2 mg. of Corper's 1305. From Feb. 5 to March 14, they were fed daily 5 mg. of thiocol and after that, 10 mg. Once a week, they were injected subcutaneously, 5 mg each time the first two weeks and 10 mg. each time thereafter. One of the pigs in this set died in 50 days; the last one died after 287 days, having received 2350 mg. of thiocol. All showed marked generalized tuberculosis, and no effect from the treatment.

Styracol Treatment.—Set 1: Jan. 3, 1917, the guinea-pigs were inoculated with 0.2 c c of "old human." Styracol is insoluble in water and the injections were therefore made in cotton seed oil, some intramuscular and some subcutaneous. These injections were made once a week and pills were fed daily except Sunday, 5 mg. for the first 2 weeks and 10 mg. from then on. The only effect of this treatment seen was an increase of generalized tuberculosis over the controls, since all 6 treated animals developed marked generalized tuberculosis while only 2 of the 6 controls showed any involvement more than a slight enlargement of the regional lymph glands.

Styracol.—Set 2: Ten guinea-pigs were inoculated with 0.2 mg. 1305. Treatment was the same as in set 1. One of the 10 guinea-pigs of this set died in 42 days with no tuberculous involvement except in the regional lymph glands, all the other pigs of this set, the last of which lived 247 days and received 1950 mg. of styracol, showed marked generalized tuberculosis, corresponding to controls given in table 6.

Orthocresol, Metacresol, Paracresol and Thymol.—Treatment: The guinea-pigs received inoculations with 0.2 mg. of culture 1305. They were fed daily 0.001 c c of the indicated drug in pill form. After the first 3 weeks, they were fed 0.002 c c daily. They also received subcutaneous injections once a week, usually 0.003 c c in water, but part of the time 0.006 c c was injected. The weekly injections and daily feedings were kept up until death. Thymol, being a solid, was weighed and 1 or 2 mg. were fed daily and 3 or 6 mg. were injected. Of the animals treated with these four drugs, none showed any effect of treatment, if we except a slight diminution of tuberculous involvement in pigs treated with paracresol and thymol. The first of the paracresol set to die lived 120 days and showed practically no generalized tuberculosis, while

even one dying after 134 days showed only a few small tubercles in the lungs, liver and spleen. The ones dying later, however, had advanced involvement of all the internal organs which are subject to tuberculous infection. Of the thymol set, 1 died in 58 days, 1 in 80 days and a third after 115 days with no or slight general involvement and 1 animal is still living and apparently well after 239 days. However, 1 dying after 136 days and 1 after 147 days showed marked generalized tuberculosis. The controls and the animals treated with orthocresol and metacresol showed without exception advanced tuberculosis, even in those dying at 73 and 87 days. The longest period of life of the controls was 171 days, of the metacresol treated, 107 days and of the orthocresol treated, 188 days.

All of the compounds of this series have shown so little local toxicity that it was at no time necessary to stop the injections on account of infiltrations, necrosis and ulcerations of the skin, and so little general toxicity that neither the weights, which were taken weekly as a guide to treatment, nor the general condition of the animals ever suggested the desirability of stopping either the feeding or the injections. Twelve normal guinea-pigs have been treated for 125 days with thymol and the cresols, using 3 animals for each drug. The same doses and method of administration have been used with these as with the tuberculous guinea-pigs. Two of these nontuberculous pigs died early from acute cage infections, but the rest are living, in good condition and gaining weight. Hence we may say that all of these drugs are relatively innocuous to guinea-pigs. We have not used intravenous injections in our experiments, since frequent intravenous injections into the same guinea-pig are difficult to make and the intracardiac injections are dangerous.

TABLE 7

Amount of Organ Involvement in Treated Tuberculous Guinea-Pigs

Control	Creosote	Guaiacol	Creosol	Thiocol	Styracol	Orthocresol	Metacresol	Paracresol	Thymol
40	55 5/7	66 1/9	68	66⅔	85				
97	98.5	88	94	89.3	87				
98⅓	.....	.....	.....	.....	.....	94	91⅔	78.6	62

In estimating the therapeutic effect of drugs, they must be judged in several ways. In human patients, it is usual to say that a drug checks or lessens cough, increases or diminishes expectoration, lessens pain and relieves other symptoms. Since experimental animals exhibit few, if any, of these symptoms, we cannot judge in this way. At the death of the animal, we can determine whether the disease is present or absent in the organs, and, if present, how its degree compares with that in control animals which died at approximately the same time after inoculation. We can also compare the duration of the disease in the treated animals with that in the control animals. In order to compare averages of these drugs with reference to the extent of the disease after their therapeutic use, we have endeavored in table 7 to represent the degree of the disease in terms of percentage, calling an extreme

involvement of all the organs 100% and so on down to 0 where all were — and then averaging these percentages.

It may be seen from table 7 that in the first set, which represented the less virulent strain of tubercle bacilli, the degree of the disease was much greater in the treated animals than in the controls, suggesting a possible stimulation of the growth or reduction in resistance. In the second set, the degree of the disease averaged less in the treated animals than in the controls, except in the case of creosote, while in the third set, the degree of the disease was much less in the treated animals than in the controls. A part of this difference may be due to earlier death of the treated animals, since the duration of life may influence the extent of the disease.

The prolongation of life, if at all marked and consistent, may also indicate some beneficial influence of a drug. Foi these reasons, it seems best, for purposes of comparison, to insert table 8 giving the average duration of life.

TABLE 8

Days of Duration of Life of Treated Tuberculous Guinea-Pigs

Control	Creosote	Guaiacol	Creosol	Thiocol	Styracol	Orthocresol	Metacresol	Paracresol	Thymol
223.2	185 4/7	285.0	134.2	146.0	150.3				
131.5	135.1	115.3	139.0	147.6	123.9				
157.5	.....	.....	.....	.....	.....	114.6	86.5	140	107.2

From all these facts, we must conclude that none of the compounds belonging to the guaiacol series, so far as we have tested them, has shown definite therapeutic action in experimental tuberculosis in guinea-pigs.

SUMMARY

Despite the extensive use of creosote and related compounds in the treatment of tuberculosis, practically no evidence exists as to the susceptibility of B. tuberculosis to antiseptics of this class, or as to their influence on the course of tuberculosis in experimental animals. A study of these problems showed that:

Virulent human tubercle bacilli are inhibited from growth (bacteriostatic action) on artificial mediums containing a concentration of 0.01% or 1 part in 10,000, each of resorcin, thymol, paracresol, orthocresol and metacresol; 0.05% (1:2,000) is the lowest concentration which completely inhibited in the case of creosol and pyrocatechin. Guaiacol, creosote, hydroquinone and guaiacol cacodylate required a concentration of 0.1% (1:1,000) to inhibit growth completely.

Sodium guaiacolate inhibited completely at 1.7%, and partially at 0.8%. Thiocol did not inhibit in 1% concentration and styracol, which is insoluble, did not inhibit in 10% concentration (suspension).

Bactericidal tests, in which the capacity to grow on agar after exposure of clumps of tubercle bacilli to the antiseptic was the measure of action, showed that the bactericidal power of these substances is low. Exposure to even 1% solutions of pyrocatechin, hydroquinone, resorcin, and 0.5% solution of creosol, for periods from 10 minutes to 48 hours, entirely fails to kill human tubercle bacilli. Metacresol and paracresol kill in 1% concentrations after exposure for one hour, but not after 10 minutes, while orthocresol reduces growth after 1 hour, and kills in 6 hours. Thymol kills even in 10 minutes at 1% concentration, but 0.1% concentration does not kill even in 48 hours. Weaker concentrations of all these antiseptics were, of course, without bactericidal effect.

A bactericidal test was made with the tubercle bacilli exposed to the antiseptics when in a thin layer on the surface of garnets, and viability determined by inoculating guinea-pigs with the treated bacilli washed from the garnets. Resorcin in 1% solution killed the tubercle bacilli only after 24 hours' exposure. Orthocresol killed in 1% concentration even in 20 minutes, but 0.5% did not kill even in 24 hours. Creosote and guaiacol both killed most of the bacilli in 0.5% concentration, even in 20 minute exposure, but 0.1% concentration was not bactericidal in 24 hours. Thymol was bactericidal in 0.1% concentration, even in an exposure of 20 minutes, but 0.01% was not bactericidal in an exposure of 24 hours.

Therapeutic tests were made on guinea-pigs injected subcutaneously with two strains of human tubercle bacilli, one highly virulent and the other much less so. The animals were then given several doses of the drug by the intracardiac, intramuscular and subcutaneous routes, and daily feedings of pills containing the drugs, the following being tested: creosote, guaiacol, creosol, thiocol, styracol, orthtocresol, metacresol, paracresol and thymol. In all, 106 guinea-pigs were thus treated for long enough periods to observe the results (besides the control experiments). Apparently the animals injected with the less virulent tubercle bacilli showed more active tuberculosis than the controls, as if the treatment had lowered their resistance or stimulated the bacilli. With the more virulent bacilli the extent of the disease was perhaps slightly less in the treated animals, probably because they commonly died a little sooner than the controls.

Our experiments show that substances of the creosote series do not possess a high bactericidal power for the tubercle bacillus in vitro, and apparently not in vivo. This result is not surprising in view of the observations of DeWitt and Sherman [30] that tubercle bacilli are rather less susceptible to fat-soluble, and more susceptible to water-soluble antiseptics, than bacteria less rich in fat than the tubercle bacillus. Also by their observation that fat-soluble dyes do not readily penetrate tubercle bacilli, while certain fat-insoluble dyes (e. g., methylene blue) stain them well. Apparently the lipin-rich character of the tubercle bacilli does not make them vulnerable to fat-soluble antiseptics, but rather the reverse.

The figures given above for the bactericidal power may be compared with those obtained by DeWitt and Sherman, using similar methods. Phenol kills in 1% concentration, and shows some effect in 0.1% concentration. Formaldehyd kills in 1% in 1 hour, in 0.1% in 24 hours. Ethyl alcohol, 25%, kills in 1 hour or less. Acetone, chloroform and ether have little or no tuberculocidal action; toluene and iodin show slight influence. Mercuric chlorid kills in 0.001% in 24 hours, 0.1% in 1 hour; gold chlorid in 0.005% kills in 24 hours, as do 0.25% silver nitrate, 0.1% gold tricyanid and 5% copper chlorid. Evidently creosote, guaiacol and the cresols, have about the same tuberculocidal power as phenol, which is distinctly not high. The dihydroxy phenols, resorcin, hydroquinone and pyrocatechin, seem to be less active than the monhydroxy phenol. Thymol was, in all experiments, distinctly, although only slightly, more bactericidal than the other substances tested. This agrees with the statement of Bechhold and Ehrlich [9] that addition of alkyl groups to phenols increases their disinfectant action.

The failure to observe any beneficial therapeutic effect on tuberculous guinea-pigs is, in view of the low bactericidal power of the substances tested, to be expected. It does not mean, however, that these substances may not have value in open tuberculous infections in man in which other bacteria than B. tuberculosis are involved. But it does substantiate the opinion that seems to have been generally reached by careful clinical observers, that creosote and guaiacol do not have a specific action on tuberculous infection.

[30] Jour. Infec. Dis., 1914, 15, p. 245.

NOMA IN THE DOG

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Fusiform bacilli and spirilla are so universally found in the morbid tissues of noma, hospital gangrene, Vincent's angina, gangrenous balanitis, and other phagadenic processes [1] that, in the light of our present knowledge, most writers believe that we are justified in considering them as the possible if not the probable causative agent in these processes. Gins [2] records the finding of bacilli identical with B. fusiformis in the intestines of lower animals, and Warthin [3] mentions finding these bacilli in the intestinal discharges of a dog affected with dysentery. We have not found, however, any previous report of a typical noma, containing these organisms, in a dog.

In a number of instances epidemics of noma have been reported as following outbreaks of other infectious diseases in institutional homes and hospitals for children.[4] Some years ago one of us saw an extensive epidemic of this kind in the diphtheria wards of the Municipal Hospital of Philadelphia, following an epidemic of measles. During the late war Campbell and Dyas [5] reported epidemics of an ulcerative condition of the mouth and throat, called "trench mouth," in which spirilla and fusiform bacilli were found in the lesions. Warthin [3] states, "Vincent's angina is regarded as directly and indirectly contagious within rather narrow limits. The affection often involves definite groups of students living in close association. A similar group-infection of nurses and hospital attendants has been observed." Such epidemics would suggest the possibility of these organisms becoming so exalted in virulence under certain conditions as to be more or less highly contagious. In fact Barker and Miller [6] state "there is positive evidence both as to the infectiousness and the

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[1] Weaver and Tunnicliff: J. Infect. Dis., 1907, 4, p. 8. King: Jour. Am. Med. Assn., 1911, 66, p. 1449. Nicoll: Archives of Pediatrics, 1911, 25, p. 912. Rothwell: Jour Am. Med. Assn., 1910, 54, p. 1867. Arrow-Smith: Annals of Otol., Rhin. and Laryng., 1910. McKinnistry: Practitioner, 1917, 99, p. 507.

[2] Handbuch d. pathog. Mikroorg., 1915, 5, p. 1007.

[3] Ref. Handbook Med. Sci., 1913, 1, p. 438.

[4] Bloomer and McFarland: Amer. Jour. Med. Sc., 1901, 122, p. 527.

[5] Jour. Am. Md. Assn., 1917, 68, p. 1596.

[6] Jour. Am. Med. Assn., 1918, 71, p. 793.

contagiousness of Vincent's disease." Therefore we consider the following case of interest as being a possible source of contagion to man.

A cocker spaniel about 9 months of age, developed a mild attack of canine distemper in March, 1918. About the time that he had apparently recovered, a peculiar and most offensive odor was noticed about the animal. Examination disclosed a tumefaction of the right lower lip as large as a quarter. On the fourth day after this an oval, black eschar appeared on its most prominent point, and salivation became profuse. An indurated, hard, dark red area surrounded the eschar. In a few days a conical ulcer formed, covered with a grayish, slimy, easily separated, and very offensive exudate. This ulcer gradually extended until on the thirteenth day of the disease it had eroded into the tissues of the neck and the ramus of the jaw, exposing the loosened molar teeth in their blackened sockets, and had attained a diameter of 3 inches. For about 8 days the general condition of the animal was astonishingly good. He ate well but seemed to suffer much from thirst. Then he became prostrated, and on the twelfth day symptoms of pneumonia developed. The dog was killed when moribund on the thirteenth day but necropsy was not obtained. On the eighth day of the disease a similar ulcer appeared on the left lip, but never became more than one-fourth the size of the original lesion.

Smears made in the early stage of the disease, soon after the appearance of the slimy exudate on the surface of the eschar, and stained with dilute carbolfuchsin, revealed the presence of about equal numbers of fusiform bacilli and spirilla, with only a few micrococci and short bacilli. The microscopic picture was striking on account of the great number of fusiform bacilli and spirilla and the small number of other organisms.

Smears were made from day to day from the lesions as the disease progressed. The microscopic picture did not vary greatly in the different preparations. The flora changed to some extent, but B. fusiformis and the spirilla greatly predominated in all smears.

A point which interested us, and one which has been noted by other observers, was the different appearance of the fusiform bacilli in the early and late stages of the disease. In the first smear these bacilli were rather uniform in size, only slightly if at all curved, and stained somewhat uniformly with dilute fuchsin. In smears made late in the disease, however, there were few bacilli of the type just described but involution forms were numerous. These stained unevenly, were much larger, and many were curved similarly to the spirilla; in fact, many long bacilli resembled thick spirilla.

Cultures were made early in the disease from the exudate in glucose horse-serum agar, both slants and stabs, and all tubes were incubated anaerobically at 37 C. for 5 days. Growth appeared in all tubes but smears from these cultures showed a mixed flora, largely micrococci. In one stab culture a few spirilla were found. In none of the cultures could we definitely identify B. fusiformis. We were inclined to interpret the few spirilla in this culture as a direct transfer of the original heavy inoculum and not a multiplication of these organisms.

Smears were prepared from the gums of a number of normal dogs in the same kennel as the case described. The majority of these smears contained B. fusiformis and spirilla, but usually in relatively small numbers. Eighteen months later the same normal dogs were examined and the results were quite similar to the first examination.

The occurrence in a dog of a noma so similar to that in man makes it appear possible that the dog may be a source of infection to man, especially to persons recovering from acute diseases who are therefore more susceptible to such infection.

In view of the fact that it has not been definitely settled whether B. fusiformis and the spirilla are symbiotic organisms or different forms of the same organisms, and further that inoculations of both the original material and pure cultures have been almost uniformly unsuccessful in producing characteristic lesions in animals, it is suggested that dogs be used for such experiments. And since noma is almost unknown in children not weakened by some previous disease, but follows various acute infections such as measles, whooping cough, etc., the chance of successfully producing noma by inoculations might be increased if dogs suffering from a mild attack of canine distemper were used in the experiment.

SUMMARY

A condition in a dog apparently identical in symptomatology and bacteriologic findings with the disease known as noma is recorded.

Attention is called to the possibility of contagion of Vincent's organisms from dogs to man.

INTESTINAL OBSTRUCTION

A STUDY OF THE INFLUENCE OF THE BACTERIAL FLORA ON THE TOXEMIA OF ACUTE OBSTRUCTION

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AND

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Complete obstruction in any region of the intestinal tract in animals produces a severe, rapidly developing toxemia. The symptoms are similar to those of acute mechanical ileus in man except that vomiting is a less noticeable feature. Death occurs in a short time after the onset of the toxemia unless the obstruction is relieved and all damaged intestine removed. It has been demonstrated both clinically and experimentally that the material which collects above the point of obstruction in the intestine is exceedingly toxic, and practically all investigators today regard the absorption of this material as responsible for the toxemia of acute obstruction. In previous studies by two of us [1] it was shown that the toxic materials in intestinal contents both under normal conditions and in cases of acute obstruction may arise independently of food and in the absence of gastric juice, pancreatic juice and bile. It was further proved that the secretions of the normal intestinal mucosa are not toxic when absorbed from the peritoneal cavity. This was confirmed by Davis and Stone [2] who noted that fresh succus entericus did not produce toxic symptoms when injected intravenously into animals. When, however, the juice was permitted to stand and the proliferation of bacteria was not prevented by heat or preservatives, it rapidly became very toxic. The production of isolated closed segments of the intestine, as described by Stone, Bernheim and Whipple,[3] produces symptoms similar to those of complete obstruction at the same level. By making use of various modifications of this procedure, we have been able to show that the presence of

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[1] Dragstedt, L. R.; Moorhead, J. J., and Burcky, F. W.: Jour. Exper. Med., 1917, 25, p. 421. Dragstedt, L. R.; Dragstedt, C. A.; McClintock, J. T. and Chase, C. S.: Jour. Exper. Med., 1919, 30, p. 109. Dragstedt, C. A.; Dragstedt, L. R., and Chase, C. S.: Am. Jour. Physiol., 1918, 46, p. 366. Dragstedt, C. A. and Moorhead, J. J.: Jour. Exper. Med., 1918, 27, p. 359.

[2] Jour. Exper. Med., 1917, 26, p. 686.

[3] Bull. Johns Hopkins Hosp., 1912, 33, p. 159.

bacteria in the lumen of the intestine is necessary for the production of the characteristic toxic substances, and in the absence of bacteria these substances are not formed. It seems highly probable that the principal toxic fraction in the contents of the obstructed intestine belongs to the group of poisonous substances which are produced by the action of bacteria on proteins or their split products. They are not toxins in the true sense of the word since they are not destroyed by heat nor will they lead to the production of antibodies when injected into animals.

The present study has been made to determine the relationship of the intestinal flora to the toxemia resulting from complete obstruction. If the toxemia is due to the absorption of poisons resulting from the action of proteolytic bacteria on proteins or their split products, it should not occur if proteolytic organisms are not present to break down nitrogenous materials.

TABLE 1

Series 1

Rat	Weight, Gm.	Diet	C-A Ratio	Days Survived Obstruction	Average Days Survived
B	130	Stock	1:99	8	
C	98	Stock	5:95	3	6.66
D	135	Stock	3:97	9	
11	150	Meat	90:10	11	
7	312	Meat	99: 1	11	8.0
15	140	Meat	91: 9	2	
12	170	Lactose	1:99	7	
2	241	Lactose	3:97	11	10.0
14	148	Lactose	1:99		

Stock Diet: oats, cabbage and carrots.
Meat Diet: groud beef, cabbage and carrots.
Lactose Diet: whole milk, whole wheat bread, lactose and cabbage.

It has been amply proved by Torrey,[5] Hull and Rettger,[6] and others, that the intestinal flora can be changed by diet. By feeding certain carbohydrates, particularly lactose and dextrin, the intestinal flora may be completely dominated by aciduric organisms with a diminution of the proteolytic types. This fact has been made the basis of the following experiments: White rats were fed on diets which changed the intestinal flora. Before operation the fresh feces from these rats were plated and the proportion of B. coli to B. acidophilus determined. This is expressed as the C-A ratio. Then under ether anesthesia a complete

[5] Jour. Med. Res., 1919, 39, p. 415.
[6] Jour. Bacteriol., 1917, 2, p. 47.

obstruction of the distal end of the cecum was made in each rat. In some of the rats the obstruction was produced by cutting the bowel, infolding and closing both proximal and distal ends. In others the obstruction was made by ligation with silk tape. The animals withstood the operation well and were eating, as a rule, within 24 hours. After operation they were fed the diets which had induced the type of flora desired.

TABLE 2
SERIES 2

Rat	Weight, Gm.	Diet	C-A Ratio	Days Survived Obstruction	Average Days Survived
13	195	Meat	94:6	2.7	2.95
17	140	Meat	80:20	2.1	
19	298	Meat	99:1	5.0	
23	174	Meat	89:11	2.0	
20	220	Lactose	3:97	16.0	8.1
24	139	Lactose	1:99	5.4	
26	184	Lactose	5:95	5.0	
28	172	Lactose	1:99	6.0	

TABLE 3
SERIES 3

Rat	Weight, Gm.	Diet	C-A Ratio	Days Survived Obstruction	Average Days Survived
37	138	Meat	99:1	8.7	8.3
51	116	Meat	50:50	9.0	
43	117	Meat	50:50	5.0	
45	166	Meat	85:15	10.5	
42	204	Lactose	1:99	9.2	7.9
44	184	Lactose	1:99	2.0	
48	166	Lactose	1:99	12.5	

The following necropsy picture was typical of all the rats: The wounds were not infected and there was no evidence of perforation or peritonitis. The cecum and small intestines were greatly distended with gas and either hemorrhagic or markedly hyperemic. The contents of the cecum and ileum were foul smelling. The indol reaction with p-dimethylamidobenzaldehyd was intense. The hydrogen sulphid odor was usually pronounced. Stains of fecal emulsions from the ileum and cecum showed a great preponderance of gram-negative organisms and culturally the C-A ratio was approximately 90:10. Even in the case of animals with an aciduric flora at the time of operation, the cecal contents at death were dominated by gram-negative proteolytic bacteria. In other words, there was a complete turnover from aciduric to proteolytic floras in cases of complete obstruction, in spite of the diets fed.

In this series the same general necropsy findings obtained as in the preceding series with the following exceptions: In rat 13, although the cecum and ileum were markedly hemorrhagic and injected and the contents were foul smelling, the indol reaction was negative. The C-A ratio was 99: 1. In the case of the lactose eaters that died the cecal flora was proteolytic, the C-A ratio being 97: 3.

The only striking feature in this series was the fact that in all the lactose eaters the C-A ratio at necropssy was reversed. The cecum and ileum were markedly hemorrhagic and distended and the indol reaction of the cecal contents was intense. In rat 44 the ligature had cut through and fecal material had penetrated into the peritoneal cavity, while in rat 42 the ileum was necrotic and easily torn.

TABLE 4

Series 4

Rat	Weight, Gm.	Diet	C–A Ratio	Days Survived Obstruction	Average Days Survived
51	170	Meat	90:10	10.0	11.5
47	200	Meat	97:3	10.0	
55	223	Meat	99:1	15.0	
53	234	Meat	95:5	11.0	
36	147	Lactose	3:97	12.75	10.0
38	137	Lactose	50:50	5.5	
30	130	Lactose	1:99	11.75	

TABLE 5

Summary of Series 1, 2, 3 and 4

	Number of Rats	Average Weight	Average Days Survived Obstruction
Meat diet	15	188.8	7.82
Lactose diet	13	172.5	8.43
Stock diet	3	121.0	6.73

DISCUSSION

In these experiments all the animals dying with symptoms of acute intestinal obstruction with greatly distended cecum and ileum had at death an intestinal flora which was markedly proteolytic. In thirteen rats whose fecal flora at the time of operation had been of the aciduric type with a great preponderance of B. acidophilus, at death B. coli had almost completely gained the ascendancy although the diet had been conducive to aciduric development in normal animals. In no case did a rat with an initial aciduric flora have this type of flora at death.

It has been demonstrated by many workers that proteolytic organisms may produce toxic substances from proteins or their split products Normally a selective protective action of the cells of the intestine probably prevents their rapid absorption. This protective action may be lost under abnormal conditions, however, as in the case of obstruction with marked distention either from gas or residue through functional or anatomic injury to the absorptive mucosa. In this case absorption of toxic substances may take place faster than the liver can detoxicate them and toxemia and death ensue.

In our experiments one fact may indicate the reason for no more striking differences in the animals operated on. It was noticeable that at the time of operation the cecums of the rats on a meat diet were very small as compared with those of the rats eating bread, milk and lactose. The cecums of the meat eaters were almost empty and averaged about 0.5 cm. in diameter, whereas those of the lactose eaters were filled with material and were frequently 2 cm. in diameter. The latter rats were obviously at a mechanical disadvantage at the outset, but in spite of this fact the average period of survival was greater than that of the meat eaters. This bulky residue may have formed a barrier which made it impossible for the lactose to keep the aciduric organisms in the ascendancy, and with the turnover to a proteolytic gas-forming flora the added distention could lead to the hemorrhagic and frequently necrotic condition of the mucosa with a consequently hastened absorption of poisonous substances.

The fact that the toxemia arising from acute intestinal obstruction is always associated with the presence of a proteolytic flora in the intestinal contents above the point of obstruction is added evidence that the responsible poisonous substances are of bacterial origin. While the work of Torrey and of Hull and Rettger is confirmed by these experiments, it is not possible to prevent the onset of toxemia in cases of acute intestinal obstruction by dietary changes calculated to substitute an innocuous aciduric flora for a proteolytic one. However, it may be possible to delay the onset of a toxemia incident to post-operative adynamic ileus by feeding lactose or dextrin before the operation and thus implant an aciduric intestinal flora. In cases of incomplete intestinal obstruction with toxic symptoms and in others in which there is reason to believe that an intoxication of intestinal origin exists but without complete obstruction, it is probable that an implantation of aciduric organisms could be secured with marked benefit.

CONCLUSIONS

The intestinal flora of white rats can be effectively controlled through regulation of the diet. The feeding of a high protein diet produces a markedly proteolytic flora while the addition of lactose or dextrin in definite amounts brings about a predominance of aciduric organisms. The work of Hull and Rettger is confirmed.

The toxemia incident to acute intestinal obstruction is uniformly associated with the presence of a proteolytic intestinal flora and that irrespective of the nature of the flora before the obstruction is produced.

It is not possible to prevent the onset of toxemia in acute intestinal obstruction in animals by feeding diets which in normal animals bring about an aciduric intestinal flora. However, the onset of the toxemia may be delayed in proportion to the degree that the aciduric flora may be maintained, presumably developing simultaneously with the appearance of a proteolytic flora.

ONCE A TYPHOID CARRIER, ALWAYS A TYPHOID CARRIER

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The part played in the incidence of typhoid fever in Massachusetts by typhoid bacilli carriers is unknown, but the number of typhoid bacilli carriers found each year is increasing, although typhoid cases reported annually in the state are decreasing in number.

The axiom of "once a carrier, always a carrier," is a good one for health officials to stand by. A series of negative fecal and urinary examinations for the typhoid bacillus should mean nothing to a board of health who are safeguarding the public health, if typhoid bacilli have been found in the excreta of a person three months or more after having had typhoid fever. It is true that occasionally the removal of a gallbladder results in negative findings. However, the mere fact that it is not true in all instances indicates that there is a chance that the bacilli are lurking in other places than the removed gallbladder, and that the person may remain a carrier indefinitely.

The small number of typhoid cases reported in recent years makes the investigation of individual cases easier than formerly when the number of cases was twice as large. A more careful investigation of the individual case for the purpose of locating the source of infection is needed in order to lower the existing morbidity and mortality rate.

Intensive work in studying individual typhoid fever cases and obtaining an epidemiologic case record for each case was started in Massachusetts in 1917. These investigations have been made by the local boards of health, state district health officers, nursing assistants and the epidemiologist. By careful questioning, the data on the case records have proved of great value in most instances, although there is an opportunity for more conscientious gathering of detailed information on the case records by some boards of health.

From the case records of the past three years, it is evident that more than half of the typhoid fever cases in Massachusetts are due to carriers. The clinical case caused 13.2% of our typhoid cases in 1918 and 1919. These far too numerous secondary cases were caused in households because physicians did not insist on administering the typhoid-paratyphoid vaccine to contacts.

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In the search for carriers it has been surprising to find the number of carriers who have no recollection of ever having had typhoid fever. This is particularly true of older persons. However, in 1919, of the 13 carriers found, only 3 gave a negative typhoid history.

Before the carrier campaign was started, it was rather unusual to find a carrier unless he was involved in an outbreak of typhoid, but this has changed, for housewives are now proving to be the most common carriers found and usually cause only one or two cases. We know that typhoid carriers caused 132 cases or 7.9% of the 1,665 typhoid fever cases studied in the state in 1918 and 1919. In addition many cases and small outbreaks were suspected of being caused by carriers, but were not proved.

The first carrier located in Massachusetts was found in 1910 and the "carrier list" now contains 51 known carriers which are given in the accompanying table.

TYPHOID CASES INFECTED BY CARRIERS

The 51 carriers were apparently responsible for 493 cases of typhoid fever. Of the 51 carriers, 21, or 41%, transmitted the infection to others by milk and 18, or 35%, were food handlers, infecting others probably by food. The remaining 12 carriers, or 24%, were in such occupations that they could have caused the cases associated with them, by food or contact.

The intermittent nature of the carrier danger is manifest in several carriers. For instance, carrier 42 caused no known cases in 1909, 1910, or the period 1914 to 1918 inclusive, yet in 1919 there were 29 cases on his milk route and for the first time, typhoid bacilli were isolated in his excreta. Similarly, carriers 8, 21 and 29 show the same peculiarity and indicate the grave menace a carrier is during his lifetime.

MILK INFECTION MINIMIZED

There would be practically no milk outbreaks of typhoid fever if we could locate the carrier before the milk was infected. For this reason it would seem advisable to have all milk handlers submit to at least three agglutination tests, fecal and urine bacteriologic examinations, even though they give no history of having had typhoid fever.

The hunt for carriers, from a financial or health standpoint, would certainly pay from an economic point of view. Once a survey of employees was completed, it would be little trouble to keep the new employees checked up.

TABLE 1

TYPHOID BACILLI CARRIERS IN MASSACHUSETTS

Carrier	Occupation	Year of Typhoid Illness	Years After Illness Before First Known Case	Cases Apparently Due to Carriers: Year	Cases Apparently Due to Carriers: Cases
1	Maid..........	No typhoid history; B. typhosus isolated in feces, 1915	..	1915	6
2	Milk handler..	No typhoid history; B. typhosus isolated in 1916.......	..	1916	9
3	Housewife.....	1914; B. typhosus isolated in feces, 1917................	..	....	..
4	Milk handler..	1870; B. typhosus isolated in feces, 1915................	45	1914	56
				1915	10
5	Waitress......	1914; B. typhosus isolated in feces, 1916................	3	1916	1
6	Waitress......	No typhoid history; B. typhosus isolated in feces, 1916	..	....	..
7	Milk handler..	No typhoid history; B. typhosus isolated in feces, 1916	..	1916	5
8	Milk handler..	"Intestinal grippe" 1896; B. typhosus isolated in feces, 1916	3?	1899	1
				1911	1
				1912	1
				1915	1
				1916	1
9	Student.......	October, 1915; B. typhosus isolated in feces, 1917.......	2	1916	2
				1917	2
10	Milk handler..	No typhoid history; B. typhosus isolated in feces, 1916	..	....	..
11	Food handler.	No typhoid history; B. typhosus isolated in feces, 1915	..	1915	22
12	Cook..........	No typhoid history; B. typhosus isolated in urine, 191	..	....	..
13	Nurse..........	No typhoid history; B. typhosus isolated in feces, 191	..	1917	1
14	Milk handler..	No typhoid history; B. typhosus isolated in feces, 191	..	1911-12	3
15	Housewife.....	1914; B. typhosus isolated in feces, 1915................	1	1915	1
16	Waitress......	1906; B. typhosus isolated in feces, 1910................	4	1909	5
17	Milk handler..	1894; B. typhosus isolated in feces, 1910................	8	1902	21
				1903	15
				1904	4
				1905	9
				1906	6
18	Milk handler..	No typhoid history; B. typhosus isolated in feces, 191	..	1913	9
19	Milk handler..	No typhoid history; B. typhosus isolated in feces, 191	..	1913	61
20	Chairmaker...	1916; B. typhosus isolated in 1916........................	..	....	..
21	Milk handler..	No typhoid history; B. typhosus isolated in feces, 191	..	1915	5
				1917	80
22	Cook..........	No typhoid history; B. typhosus isolated in feces, 191	..	1916	5
				1917	1
23	Student.......	1917; B. typhosus isolated in feces, 1917................	6 mo.	1917	1
24	Milk handler..	No typhoid history; B. typhosus isolated in feces, 191	..	1917	4
25	Housewife.....	1913; B. typhosus isolated in feces, 1917................	4	1917	1
26	Unknown.......	July, 1917; B. typhosus isolated in feces, Jan., 1918.....	..	....	..
27	Laborer.......	June, 1917; B. typhosus isolated in urine, Nov., 1917....	5 mo.	1917	2
28	Iceman........	Sept., 1915; B. typhosus isolated in Dec., 1915...........	3 mo.	1915	2
29	Milk handler..	1909; B. typhosus isolated in 1918........................	9	1911	10
				1914	2
				1915	1
				1918	2
30	Maid..........	1895; B. typhosus isolated in 1918........................	23	1918	1
31	Milk handler..	1917; B. typhosus isolated in feces, 1918................	1	1918	6
32	Milk handler.	No typhoid history; B. typhosus isolated in urine, Aug., 1918	1	1918	2
33	Milk handler..	No typhoid history; B. typhosus isolated in feces, Sept., 1918	1	1918	3
34	Laborer.......	1916; B. typhosus isolated in feces, 1918................	2	1918	1
35	Milk handler.	No typhoid history; B. typhosus isolated in feces. 1919	..	1919	8
36	Student......	Feb., 1919; B. typhosus isolated in feces, Aug., 1919....	6 mo.	1919	1
37	Milk handler.	1913; B. typhosus isolated in urine, 1919................	6	1919	8 ?
38	Maid.........	1914; B. typhosus isolated in feces, 1919................	5	1919	1
39	Milk handler.	No typhoid history; B. typhosus isolated in feces, 1919; found in same outbreak as Carrier 37	..	1919	8
40	Mechanic.....	1918; B. typhosus isolated in feces in June, 1919........	1	1919	1
41	Milk handler.	1898; B. typhosus isolated in feces, 1918................	20	1918	6
42	Milk handler.	1906; B. typhosus isolated in feces, 1919................	13	1907	5
				1908	2
				1911	2
				1912	2
				1913	33
				1919	29
43	Milk handler.	1915: B. typhosus isolated in feces, 1919; this carrier caused other deaths in N. H.	4	1919	3
44	Housewife.....	No typhoid history; B. typhosus isolated in feces, 1919	..	1918	2
				1919	2
45	Housewife.....	1901; B. typhosus isolated in feces, 1919................	18	1919	1
46	Housewife.....	Oct., 1918; B. typhosus isolated in feces, Feb., 1919.....	4 mo.	1919	1
47	Manager......	1903; B. typhosus isolated in feces, 1919................	16	1919	1
48	Cook..........	1915; B. typhosus isolated in feces, 1919................	4	1919	1
49	Maid..........	1915; B. typhosus isolated in feces, 1920................	5	1919	1
50	Housewife.....	1913; B. typhosus isolated in feces, 1920................	7	1920	1
51	Caretaker.....	Nov., 1919; B. typhosus isolated in feces, March, 1920..	..	....	..

PASTEURIZATION

Pasteurization has undoubtedly prevented outbreaks of disease, but an outbreak of septic sore throat occurred in Massachusetts on a route on which pasteurized milk was supplied, because the milk was infected after pasteurization. There is an opportunity for infection in the process of capping the bottles and it would seem desirable to have employees doing this work examined in order to discover whether they are carriers.

TABLE 2

Summary of Carriers and Cases They Infected

	Number of Carriers	Typhoid Cases
Milk handlers	21	426
Food handlers		
Cooks	4	29
Maids	7	15
Housewives	7	9
Nurses	1	1
Icemen	1	2
Nonfood handlers	10	11
Total	51	493

OCCUPATIONS

It is rather startling to find typhoid carriers among nurses, but two were found. One who handled milk caused 6 cases and the other infected her patient. In recent years it is the houseworker, the maid, waitress, cook or wife who causes the carrier cases and it is a good indication of the activity of the local boards of health along the line of communicable disease prevention that such carriers have been found.

LENGTH OF CARRIER STATE

The question is often asked in regard to the length of time a person may be a carrier and the only answer is "always."

One carrier had typhoid 44 years prior to the first case traced to him, and others 20, 18, 16 years and shorter periods.

The time interval was not obtained for all carriers because of the inability of the carrier to supply reliable data.

RELEASE CULTURES IN CLINICAL CASES

A system of examining the urine and feces of each typhoid patient at hospitals and requiring three negative findings on three specimens taken at least 48 hours apart before discharging the patient, brought to light three carriers in two hospitals during 1918 and 1919.

This method of "release cultures" should prove of the greatest value in locating carriers before they cause infection rather than after, as does the present method.

CHECKING OF CARRIERS

At four-month intervals investigations are made to determine whether each typhoid carrier in the state has "kept faith" and is not engaged in an occupation in which food is handled or in an occupation whereby the public health would be endangered. Up to the present time, no typhoid carriers have been known to have caused an infection, since being proved carriers.

LABORATORY PROCEDURE

The bacteriologic laboratory distributes a double mailing case enclosing a rubber stoppered test tube containing 30% glycerol in 0.6% sodium chlorid solution. This outfit is used for sending specimens of feces and urine to the laboratory. The amount of feces sent is, as a rule, about one-fifth of the total volume of the emulsion and the amount of urine about one-half. This outfit was designed primarily for feces specimens as the majority of typhoid carriers are fecal carriers. The glycerol solution recommended by Teague and Clurman has been found most valuable for the preservation of specimens delayed in transit.

As soon as the specimen arrives at the laboratory it is streaked with a platinum loop on large (13 cm.) plates of Endo's medium, freshly prepared each day. The eosin-methylene blue agar of Holt-Harris and Teague is also used. For specimens of feces two plates of each medium are used and two or three loopfuls of the feces suspension are used to a plate. For urine, approximately 20 loopfuls to a plate are used.

After 18-24 hours' incubation at body temperature, 6 or more colonies resembling B. typhosus are fished and each inoculated into 2 c c nutrient broth. Should no suspicious colonies be seen but many colonies of the colon group, fresh plates are streaked with the specimen, then 24 hours older. It has been found that glycerol inhibits colon bacilli more than typhoid and that an older specimen showing fewer colon colonies on a plate occasionally gives positive results because there is less overgrowth of the typhoid bacilli with other organisms.

After 4-6 hours' incubation at 37 C., the broth cultures are examined in hanging drop for motile bacilli resembling typhoid. If any are found, they are mixed with a typhoid agglutinating serum, in appropriate dilution, and examined within an hour for agglutination. If one of the cultures is completely agglutinated it is inoculated into dextrose and lactose broths in fermentation tubes. A culture forming acid in dextrose broth and no gas in either sugar broth within 48 hours is reported as B. typhosus.

BACILLUS PERFRINGENS: TOXIN AND ANTITOXIN PRODUCTION

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With few exceptions the reports on the bacteriology and immunology of gas gangrene have given only few details regarding the methods found most advantageous for the production of toxin and antitoxin. Therefore it seemed worth while to record certain observations made in the production and standardization of an antiserum to B. perfringens and a combined antiserum to B. perfringens and B. tetani.

Cultural Methods

The detailed experiments of Bull and Pritchett[1] on the factors influencing the artificial production of toxin showed clearly, among other features, the effect of the incubation period, the addition of fresh muscle and of glucose. At first I found the addition of fresh muscle to be sufficiently troublesome to induce one to determine whether it was worth the time necessary, in view of the purpose of the work. It had appeared early in the work that toxin production was closely proportional to infectivity of the culture. Subsequent work appeared to support this conclusion although definite experiments were not planned to determine the exact relationship between these two phenomena. However, they were sufficiently closely related, and the difficulty of maintaining the virulence of the cultures sufficiently marked to make it advisable to investigate any methods whereby the infectivity of the strain might more easily be maintained.

Fresh muscle glucose (0.2%) broth and autoclaved muscle glucose (0.2%) broth cultures were contrasted regarding their infectivity. Six pigeons varying in weight from 300 to 340 gm. were used; 3 of these received fresh muscle broth culture intramuscularly in the following doses, 0.1 c c, 0.5 c c and 0.01 c c; the remaining 3 received the autoclaved muscle culture in the following doses: 0.05 c c, 0.01 c c and 0.005 c c. These cultures were approximately 20 hours old, had been seeded with equal amounts from the same parent culture, and the inoculations were given at 4 p. m. By next morning the 3 pigeons that had received the fresh muscle culture were dead, while the remaining 3 birds all survived, though they showed varying degrees of focal lesion according to the size of the dose.

This experiment had been planned to show whether the effect of adding fresh muscle to the mediums was sufficiently important to make its adoption as a routine measure advisable, and also to insure a successful passage, as at that time the virulence of the culture used had dropped to a low point. This result was so much more favorable for the fresh muscle medium that its use was adopted as a routine measure.

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[1] J. Exper. Med. 1917, 26, p. 119, 603, and 867.

This is the technic finally adopted for the preparation of fresh muscle broth: A healthy rabbit was chloroformed, the back and thigh shaved, and the skin thus prepared scarred with a red hot iron. Knives, long handled scissors, and long forceps were dry sterilized. The back muscles, one side at a time, exposed and appropriate sized pieces of muscle cut and deposited in wide mouthed bottles containing sterile salt solution. It was only with considerable difficulty that freshly cut pieces of muscle could be inserted into even wide culture tubes, because they tend to stick to the sides of the tubes wherever they touch the glass. This difficulty was found to be almost entirely overcome by the use of the salt solution. Throughout the operation the free bunsen flame was lightly and occasionally used on the exposed muscles and for the instruments. The charred pieces of muscle which became attached to the instruments were detached by wiping with small moist pads of sterile cotton wool. When a sufficient number of pieces had been deposited in the bottles of salt solution they were transferred piece by piece into the tubes or flasks of medium, the bottles and medium containers being slightly slanted to allow the use of the free bunsen flame as desired. The time required after the rabbit was shaved to complete the preparation of the medium was about an hour. Incubation for 48 hours for sterility showed that usually less than 1% of the tubes would be contaminated although on two or three occasions about 40% were not sterile. The percentage of contamination for Erlenmeyer flasks of broth was always two or three times that of the tubes.

To prevent evaporation it was found advisable to add sterile paraffin oil to all but a few tubes which were kept for early use, and to incubate again for sterility. The paraffin oil was sterilized in a flask so equipped that the oil could be syphoned off.

Pasteur pipets were used for transplantation from the oil covered cultures, not only because in this way the oil could be avoided and films made, but also because large amounts could be carried over.

The anaerobic method found most suitable was the simple one used by Bull and Pritchett. The oil covered tubes or flasks, after being inoculated, were enclosed in a vacuum jar from which the oxygen was then exhausted. The additional use of pyrogallic acid and KOH was not found to have any advantage.

Contamination, which was liable to appear in the cultures made from the pigeons or guinea-pigs used as a means of maintaining virulence, was controlled by seeding on a slope agar and incubating aerobically. This, and films from the broth culture, proved to be a satisfactory routine method for the control of this factor. The need for the early detection of contamination arose from the difficulty of maintaining the virulence throughout the procedures necessary to obtain again the B. perfringens in pure culture when once contaminated. Rather than resort to this it was found that time was saved by carrying on from an older culture. If a contaminated culture had to be used, frequent subculturing (after 6-8 hours' growth anaerobically) seemed to be the most satisfactory method of again obtaining the bacillus in pure culture without the loss of virulence that invariably accompanied any method involving the use of solid medium.

Bull and Prichett have commented on the aggressin like action of B. perfringens in that the pathogenicity of other micro-organisms is increased when present in the host experimentally infected with B. perfringens. This appeared to play a rôle in my hands on several

occasions. Such a phenomenon opens up several interesting paths that might prove of value in the investigation of other infections, particularly those of the subacute varieties. The observations on the bacterial flora found in association with B. perfringens in war wounds when the toxemia of the patient was marked, although cultures of B. perfringens showed but slight pathogenicity, may well be due, to a great extent, to this characteristic.

Maintenance of Virulence

As already commented on, there was every indication that the potency of the filtrate (toxin) was proportional to the infectiousness of the culture. Unless the utmost care was used great loss in the virulence of the strain would occasionally show itself in the more or less routine fashion in which pigeons were used to maintain this, and considerable loss of time was occasioned in raising the virulence. Under the most careful technic contamination would appear in the cultures made from animal or bird passage. This apparently occurred more readily when guinea-pigs were used instead of pigeons.

On one of these occasions, with the hope of more rapidly increasing virulence, bird to bird passage was tried under the following circumstances: Culture 617 D had lost virulence to such an extent that the inoculation of 0.1 c c of an 18-hour, fresh muscle glucose-broth culture into the breast muscles of a 310 gm. pigeon showed after 24 hours no general intoxication but considerable local swelling and gas infiltration. The pigeon which apparently was likely to recover was chloroformed, the lesion massaged and bruised so that it was possible to withdraw aseptically by means of a syringe and wide bore needle 0.8 c c of thick blood stained semifluid débris. Films made from this showed broken down muscle tissue, blood cells, débris and an average of from 20-40 clearly outlined bacilli per field. With this material two pigeons were inoculated: One pigeon (310 gm.) received the entire 0.8 c c, and the other (290 gm.) the washings obtained by rinsing the syringe with 0.5 c c sterile salt solution. The pigeon which had received the undiluted dose showed a barely perceptible local and no general reaction, while the pigeon that received the diluted material showed no effects. The virulence of this strain was, however, increased by inoculating birds with massive doses of subcultures originating from this same pigeon.

Every attempt to raise the virulence by direct passage methods failed although the virulence was on each of these occasions increased by cultural methods.

I do not mean to suggest that virulence, under appropriate and favorable circumstances, can not be maintained or even increased by direct passage from bird to bird; but the fact that direct passage can fail in these respects provides, I think, a further suggestion regarding the manner in which B. perfringens produces its pathogenic effects, which I would enlarge on as follows:

It has been previously mentioned that, as a routine control against contamination, slope agar cultures were made and incubated aerobically. It was noticed that frequently after 36 hours a fine film could be detected on the surface of the agar which on staining proved to be gram-positive bacilli, the majority of which were longer and finer than when grown anaerobically. If left in the incubator or even in room temperature these cultures often showed fairly luxuriant growth. A thick emulsion in salt solution from two such 52-hour agar cultures failed to produce any gross local lesion when inoculated into the breast muscles of a pigeon, although the 18-hour muscle glucose broth culture to which these agar cultures acted as controls killed a pigeon in a dose less than 0.01 c c in 8 hours.

When these results are viewed in the light shed by the apparently similar effects, local and general, that the inoculation of whole culture and filtrate (toxin) will regularly produce (except the gas infiltration), they emphasize

the conclusion reached by Bull and Pritchett that exotoxin, which the Welch bacillus produces under suitable conditions of growth, by itself can act on the tissues in a manner identical with the action of the whole cultures. It would further appear that the bacilli if injected by themselves and not in combination with exotoxin may prove nonpathogenic whether the bacilli had previously been separated from their exotoxin by centrifuging and washing or grown in such a fashion that toxin was not produced either in vivo or in vitro.

The detailed results obtained by Bull and Pritchett on the influence of different mediums and the period of incubation with regard to the potency of the toxin allowed them to summarize these factors in the statement "that toxicity of the filtrates is inversely proportional to the incubation time calculating from the end of the first day, and that this general relation obtains independently of the nature of the medium. . . . The rapidity of the decrease in toxicity, however, is materially influenced by the percentage of glucose in the medium and the presence of raw muscle. In my hands it became increasingly noticeable that a decrease of the infectivity of the culture began before 24 hours.

When filtration of the culture was desired it was found difficult to get sufficient material from the killed pigeon to inoculate the necessarily large amounts of medium required and on this account a short incubation of from 6-8 hours was tried with culture tubes heavily planted from the bird lesion. Luxuriant growth took place in this time and provided an excellent parent culture for the larger amounts of medium designed for filtration. If the pigeon inoculation was so timed that death took place during the night the intermediate culture tubes could be planted early in the morning and used to inoculate the larger amounts of medium late that afternoon, so that filtration could be started early next day after from 15-18 hours' (or even less) incubation.

By the injection of pigeons with these short incubation cultures it was found that even 6-8 hours' incubation produced a culture of high virulence. This rapid production of toxin was not observed until late in the course of the work and consequently was not as fully investigated as its importance warranted both for practical and academic purposes. Previous to these observations the routine results had continuously led to shortening of the incubation time. It was to some extent on this account that the use of the intermediate culture was tried because when large amounts of medium were inoculated directly from the bird lesion luxuriant growth frequently failed to take place within 18 hours' incubation. Filtration of such insufficiently grown cultures gave a toxin of low potency.

Acting on the suggestion provided by the demonstrated infectivity of 6-8 hour cultures the virulence of the strain was easily maintained (in contrast to the earlier experience) between the time when the horses were bled and the final testing was carried out. By this method one had a cycle of bird subculture and bird incubation with ensuing death in 24 hours.

As noted by other workers, considerable loss of toxicity took place during the process of filtration, so that the time involved was shortened as much as possible by the following technic. The oil covered cultures were passed through a paper mash which freed the fluid from all broken bits of muscle tissue, and if stopped before any of the oil passed through, a fairly clear and oil free fluid was obtained. This was filtered through a medium pore Berkefeld candle and collected in sterile colored glass bottles containing a few cubic centimeters of sterile paraffin oil.

It was tentatively assumed that for immunization purposes the injection of a filtrate of high potency rather than a larger amount of filtrate of lower virulence (though the two amounts might represent the same number of M L D's) would be more likely to produce an antiserum of higher protective strength.

On this conception cultures were not used for filtration unless at least 0.02 c c of the supernatant fluid produced death in a pigeon weighing from 300-350 gm. in 10-12 hours. From the literature, I have gained the impression that this was easily obtained but in my hands I found that considerable care had to be exercised to keep the virulence up to this point, and further, that considerable time would be lost before the virulence could be raised again once this was allowed to sink to such an extent that over 1 c c of the culture had to be injected intramuscularly into the pigeon to cause death.

STANDARDIZATION OF ANTISERUM

The Bull-Pritchett method of estimating the antitoxic titer of the serum to be tested was essentially the determination of the amount of serum necessary to completely neutralize 1 M L D of toxin. The various serum dilutions and 1 M L D toxin were incubated for one hour and then injected into the breast muscles of the pigeon. Absolute neutralization as shown at the site of inoculation was the criterion by which this was estimated. By this method one was occasionally left in doubt as to which bird showed absolute neutralization, and it seemed advantageous that the determination of this point should not rest on an opinion but on some fact. To realize this, 2 M L D's of toxin were used as the test dose and the smallest amount of serum which would allow the bird to survive 36 hours was determined. The serum-toxin mixtures were incubated for one hour and the smallest amount of serum required to save the life of the bird estimated as 1 antitoxin unit.

Dr. G. W. McCoy of the United States Public Health Service was kind enough to forward to me the regulations contemplated by his department regarding antiserum against B. perfringens. Their method of standardization suggested that 10 M L D's of toxin be used as the test dose against which the serum to be tested should be diluted to at least 1 in 100. The dilution affording protection against this test toxic dose was called one antitoxic unit. Consequently, 1 c c of an undiluted serum fulfilling these requirements represented protection against 1,000 M L D's of toxin. They further proposed to make it essential that 1 c c of an acceptable serum should have an antitoxic value of 10 such antitoxic units. There seemed to be no particular advantage in using 10 M L D's while at the same time it necessitated the injection of five times the amount of toxin-antitoxin mixture. Pigeons of a weight close to 325 gm., or as recommended by Bull and Pritchett, 350 gm., seemed to give the most consistent results. The results obtained with birds weighing 300 gm. were liable to give somewhat erratic results but had frequently of necessity to be used. Under the circumstances the use of two pigeons per dose was an advisable procedure, as suggested by the United States Public Health Service.

PROTECTIVE VALUE OF ANTISERUM

The characteristic lesions following the inoculation of animals and birds with cultures of the different anaerobes recovered from gas wound infection have been so fully described by most of those who have worked on gas gangrene that it seems superfluous to add to the descriptions already given. Bull and Pritchett in particular drew attention to the fact that with B. perfringens the causative factor was probably wholly due to the exotoxins and that, except for the gas formation, the toxins[2] and the whole culture produced practically identical results.

[2] Pease, Marshall C.: Proc. Soc. Exper. Biol. and Med. 1919, 17, p. 30.

Weinburg and Seguin,[3] in their most exhaustive work, and Sacquepée,[4] in his various publications, have dealt extensively with the pathogenicity of the various anaerobes and the types of lesion that can be produced experimentally by each or by various combinations of the different anaerobes.

For the purpose for which this work was begun it was thought sufficient to disregard this phase in view of the results obtained by Bull and Pritchett after the following experiment (table 1) which was designed roughly to demonstrate whether the antitoxic serum was protective alike to whole culture and to toxin filtrate.

TABLE 1

Results of Experiment to Show Protective Power of Antitoxic Serum for Whole Culture and Toxin Filtrate

Pigeon	Toxin per 100 Gm., c c	Toxin per Pigeon, c c	Whole Culture per Pigeon, c c	Prophylactic Injection Antitoxin 3 Days Before, c c	Therapeutic Injection Antitoxin After 5 Minutes, c c	Result: After 4 Hours	Result: Later
839	0.02	...	....	...	...	Sick	Died emaciated 21 days later
9839	0.04	...	....	...	...	Very sick	Survived
838	0.06	...	....	...	. .	Very sick	Died during night
26	0.08	...	....	...	...	Very sick	Died during night
4463	0.1	...	....	...	...	Moribund	
8826	0.2	...	....	...	...	Dead	
834	....	...	0.25	...	...	Dead	
842	....	...	0.15	...	...	Dead	
835	....	...	0.05	...	...	Dead	
476	....	5.0	....	2.0	...	Alive and well	No effects
479	....	1.0	....	2.0	...	Alive and well	No effects
480	....	...	0.25	2.0	...	Alive and well	No effects
843	....	2.0	....	...	2.0	Alive and well	No effects
840	....	0.5	....	...	2.0	Alive and well	No effects
841	....	...	0.25	...	2.0	Alive and well	No effects

Weinberg and Seguin describe three types of antiserum to B. perfringens, namely: a polyvalent antibacterial serum, a monovalent antibacterial serum and an antitoxic serum. The first was prepared by inoculating the horse with increasing doses of bacilli freed from toxin by washing and then continuing with increasing doses of whole fluid culture. The second was prepared by inoculating with increasing doses of bacilli freed from toxin by washing and for this serum the most virulent strain available was used. They report that with this latter strain the horse developed severe reactions—temperature 39 C., edema of the legs, etc. The third, namely antitoxin, was apparently not actually produced as under this heading it is stated that "the production is dependent on obtaining regularly a potent toxin and that the problem is associated with a number of difficulties."

[3] La Gangrene Gazeuse, 1918.

[4] Presse méd. 1918, 22, p. 197.

The most effective antiserum was the monovalent which appeared to protect against at most, 2 M L D's of toxin in a dilution of 1:200.

According to the deductions which my results would lead me to make even this degree of antitoxin production resulted from the presence of toxin which was either carried over with the bacilli or produced by the bacilli after they had been injected into the host, to which latter possibility the severe reactions would lend some support. The particular cultures used would appear to be less virulent than I found it advisable to employ for immunization purposes as the M L D was at the best 0.5 c c.

Certain features which in the literature at my disposal I have not seen commented on, and which would seem to be of sufficient importance to be recorded may be summed up thus:

With any fairly potent toxin according to the size of the dose it would seem possible to produce death in any desired space of time from about 5 minutes to 24 hours, after which it is problematical whether the pigeon will permanently recover with the extrusion of a hard calculus-like mass or continue in a state of emaciation and eventually die in from 6 weeks to 3 months.

Following the inoculation of either whole culture or filtrate, death usually takes place within 12 hours, rarely after 24 hours unless it be the greatly delayed death of several weeks' duration, when emaciation has become marked.

No practical difference has been noted between the deaths due to whole culture and toxins, unless it be in the instances when survival took place, that this usually became apparent in the case of toxin injection sooner than when whole cultures were inoculated.

When death took place, after 5-8 hours, it was always initiated by loss of balance so that if one lightly touched the pigeon it would fall over. This method was noted as a routine and death can be predicted almost to the hour from this sign. The possibility of this being due to a definite fixation of the toxin by certain of the body cells (analogous to the fixation which takes place with tetanus toxin) was not investigated. A certain amount of this assumed fixation, when toxin alone was injected, could be observed with recovery. If, however, this sign became marked death was certain.

The interval between the time of the inoculation and death in the pigeon is rapidly shortened by comparatively slight increase in the dose. Thus, toxin 57, which at 0.12 c c per 100 gm. pigeon caused death within 24 hours, killed in from 5-10 minutes when the dose was increased to 1.2 c c per 100 gm. pigeon. The weights of these two pigeons were 300 and 320 gm., respectively. Intermediate doses caused death between these two extremes of 10 minutes and 24 hours.

Immunization of Horses

Immunization of two horses against B. perfringens toxin was begun June, 1918, and continued by increasing doses until November when they were bled, seven days after the last injection of toxin. In August, 1918, an attempt was begun to obtain a combined antiperfringens and antitetanus serum by giving two horses, for some time under active immunization against B. tetani toxin, additional injections of increasing amounts of B. perfringens toxin. At first the perfringens toxin was administered rather conservatively for fear of producing too violent reactions. However, as this did not follow the dose was increased and the interval shortened, the highest temperature reaction obtained being 104 F. No lasting local reaction or noticeable edema of the legs occurred throughout the course of injections.

The two horses on B. perfringens, 2 G and 4 G, received, respectively, a total of 1820 c c and 1760 c c. The two horses 36 and 38 on combined injections received 955 c c and 970 c c, respectively. The toxin varied in potency for the different injections between a M L D of 0.04 c c to 0.1 c c per 100 gm. pigeon. The increase of dose was judged by the M L D potency and not by the total amount in cubic centimeters.

TABLE 2

Toxin Titrations

Pigeons	Weight in Grams	Toxin per 100 Grams of Pigeon, c c	Total Toxin Injected, c c	Result
A	320	0.05	0.16	Survived
D	290	0.06	0.17	Died during night
F	340	0.07	0.24	Survived
B	300	0.08	0.24	Died during night
E	300	0.09	0.27	Died during night
C	310	0.1	0.31	Died during night

TABLE 3

Standardization of Antitoxin

Pigeon	Weight in Grams	Toxin, 2 M L D per 300 gm. Pigeon	Toxin, Actual Dose in c c	Number of Horse (Antitoxin)	Antitoxin per 300 gm. Pigeon	Antitoxin, Actual Dose in c c of	Dilution	Result
31	365	0.39 c c	0.474 c c	2G Conc.	0.0005 c c	0.608 c c of	1:1000	Surv.
27	305	0.39 c c	0.396 c c	2G Conc.	0.0004 c c	0.405 c c of	1:1000	Surv.
33	310	0.39 c c	0.403 c c	2G Conc.	0.0003 c c	0.310 c c of	1:1000	Surv.
32	245	0.39 c c	0.318 c c	2G Conc.	0.0002 c c	0.163 c c of	1:1000	Surv.
28	275	0.39 c c	0.359 c c	2G Conc.	0.0001 c c	0.093 c c of	1:1000	Surv.
30	345	0.39 c c	0.448 c c	2G Lymph	0.0005 c c	0.575 c c of	1:1000	Surv.
34	385	0.39 c c	0.50 c c	2G Lymph	0.0004 c c	0.513 c c of	1:1000	Surv.
29	395	0.39 c c	0.513 c c	2G Lymph	0.0003 c c	0.395 c c of	1:1000	Surv.
41	285	0.39 c c	0.37 c c	2G Lymph	0.0002 c c	0.190 c c of	1:1000	Surv.
37	295	0.39 c c	0.38 c c	2G Lymph	0.0001 c c	0.098 c c of	1:1000	Surv.
51	250	0.39 c c	0.32 c c	2G Conc.	0.0002 c c	0.83 c c of	1:5000	Surv.
52	270	0.39 c c	0.34 c c	2G Conc.	0.0001 c c	0.45 c c of	1:5000	Surv.
53	270	0.39 c c	0.34 c c	2G Conc.	0.00006 c c	0.29 c c of	1:5000	Surv.
54	280	0.39 c c	0.36 c c	2G Conc.	0.00005 c c	0.23 c c of	1:5000	Surv.
55	260	0.39 c c	0.33 c c	2G Lymph	0.0002 c c	0.866 c c of	1:5000	Surv.
56	260	0.39 c c	0.33 c c	2G Lymph	0.0001 c c	0.43 c c of	1:5000	Surv.
58	280	0.39 c c	0.36 c c	2G Lymph	0.00006 c c	0.308 c c of	1:5000	Surv.
59	250	0.39 c c	0.32 c c	2G Lymph	0.00005 c c	0.208 c c of	1:5000	Surv.
23	300	0.39 c c	0.39 c c	2G Conc.	0.00005 c c	1.0 c c of	1:20000	Surv.
7	360	0.39 c c	0.47 c c	2G Conc.	0.000025 c c	0 6 c c of	1:20000	Surv.
10	290	0.39 c c	0.38 c c	2G Conc.	0.00001 c c	0.19 c c of	1:20000	D. 24 hr.
11	250	0.39 c c	0.32 c c	2G Lymph	0.00005 c c	0.83 c c of	1:20000	D. d. n.
12	250	0.39 c c	0.32 c c	2G Lymph	0.000025 c c	0.42 c c of	1:20000	D. d. n.
13	280	0.39 c c	0.36 c c	2G Lymph	0.00001 c c	0.19 c c of	1:20000	D. d. n.

Pigeon	Weight in gm.	Toxin 136 per 100 gm. Pigeon	Actual Dose of Toxin	Result
3	255	0.05 c c	0.13 c c	Survived
1	250	0.06 c	0.015 c c	Died during night
2	260	0.07 c c	0.18 c c	Died during night

Surv. = survived; D. = died; D. d. n. = died during night.

A trial test of the two horses 2 G and 4 G on August 23, 1919, showed neutralization by the method of Bull and Pritchett, for the 2 G serum in a dilution of 1:600 to 1:800, and for the 4 G serum in a dilution of 1:600.

The protocol for standardization of antitoxin suggested by the United States Public Health Service corrected the actual dose of antitoxin as well as toxin according to the weight of the bird. This seemed a desirable modification and was thereafter adopted.

The final standardization of the antiserum was postponed until both serum, concentrated by the method of Banzhaff, and Lymph could be contrasted.

In the standardization of toxin slight irregularities were occasionally encountered, so that one might be left in some doubt regarding the exact dose which should be considered the true M L D for the subsequent standardization of the antiserum. The use of a 2 M L D amount as the test dose has a practical value in that any error, made in the estimated M L D, will be exposed by the additional controls made in the standardization of antitoxin experiment and can be taken into account. The standardization of the antiserum was proceeded with as soon as the M L D of the test toxin was obtained on account of the possible deterioration of the toxin.

Table 2 shows the results of the titration of toxin, and table 3, the method adopted for routine for standardization of antitoxin.

Table 4 shows the results obtained for eight samples. For the data on the antitetanic serum I am indebted to the Antitoxin Division of the Connaught Laboratories.

TABLE 4

Experiment on Four Horses with Perfringens Toxin Alone and Combined with Tetanus Toxin

Antitoxin	Smallest Amount of Antitoxin (per 300 Gm. Pigeon) Affording Protection Against 2 M L D's Toxin	Number of Antitoxin Units per c c (perfringens)	Number of Antitoxin Units per c c (tetanus)
2G concentrated	0.000025 c c	40,000	
2G lymph	0.000066 c c	15,000	
4G concentrated	0.000025 c c	40,000	
4G lymph	0.00005 c c	20,000	
36 concentrated	0.0001 c c	10,000	700
36 lymph	0.0002 c c	5,000	150
38 concentrated	0.000066 c c	15,000	700
38 lymph	Less than 0.0005 c c	2,000 (?)	150

DISCUSSION

With the cessation of the war the urgent necessity for the production of antiserum against gas gangrene has largely subsided. However, a sufficient number of cases are encountered in civil practice to warrant further work both for practical and scientific purposes.

The large amount of routine and experimental work carried on especially during the latter years of the war has given us a fairly well accepted identification of the bacterial flora, anaerobic and aerobic, encountered in gas gangrene following war wounds, has separated

those essentially important etiologically, and has shown that experimentally the different infections can be prevented by their specific antitoxins, and can even be successfully treated after infection, if the interval, before treatment is begun, is not too long delayed.

Van Beuren [5] in a clinical review states that "while serum therapy promises much for the future it has not had much opportunity for performance in the past, or if it has had the reports are not yet available."

Weinberg and Seguin [3] give full details in 30 controlled cases which received specific treatment. Of these, 11 died and 19 recovered. The 11 instances of mortality are divided into 3 groups as follows:

1. The antiserum used did not correspond to the anaerobe found by bacteriologic investigation in 3 cases.

2. The proper specific serum was employed but treatment was begun too late in 5 cases.

3. The proper specific serum was employed and apparently had some effect on its specific infection but death followed from other complications in 3 cases.

The 3 antiserums used were antiperfringens, anti-oedematiens, and antiseptique. The antitoxic titer is not given, but the amounts of serum injected varied from 20 to 50 c c daily or oftener. The inoculations were given both subcutaneously about the wound and intravenously.

If one accepts B. bellonensis (B. de l'oedeme gazeus malin) of Sacquepée as identical with the B. oedematiens, as is admitted probably by both Weinberg and the Medical Research [6] Anaerobic Committee, practically all observers are in accord regarding the etiologic rôle of B. perfringens, B. oedematiens and V. septique, though Sacquepée would appear to question the essentially etiologic importance of B. perfringens chiefly on these grounds:

1. Cultures and films taken from guinea-pigs inoculated with a mixture of V. septique and B. perfringens showed that B. perfringens had grown much more luxuriously than V. septique, both in the host and the cultures; however, when inoculated separately, V. septique had been demonstrated to be much more virulent.

2. He considered these observations to be a probable explanation of the greater frequency with which B. perfringens was recovered from war wounds, and that though important, its pathogenicity came into play as a secondary invader.

[5] Jour. Am. Med. Assn., 1919, 73, p. 239.

[6] Medical Research Committee Reports, Classification and study of the anaerobic bacteria of war wounds. Series No 12, 1917.

3. Pathogenicity was frequently lessened and variable as noted experimentally with B. perfringens in contrast with V. septique or B. oedematiens.

Regarding the frequency with which only one strain could be identified, Weinberg reports the following results for the 12 cases in which monoinfection was found in his series: B. perfringens 9 times, B. oedematiens twice, and B. fallax once.

Judging from the extreme ease and rapidity with which the two strains of B. perfringens, 617 D and B 2, would fail to show pathogenicity because the production of exotoxin was inhibited either in vivo and in vitro, these observations are not to my conception contradictory to the importance of B. perfringens etiologically, but support the idea that the environment, be it due to lacerated tissue,[7] other micro-organisms or any favorable cause, must be such as to encourage the production of exotoxin before B. perfringens exerts its pathogenic effect.

In this connection, an interesting observation was made by Weinberg and Seguin regarding the action of the filtrate of B. sporogenes on the toxin of B. oedematiens, V. septique and B. perfringens. They were able to demonstrate that while the toxin of B. perfringens was unaffected by incubation with the filtrate of B. sporogenes, the toxins of both B. oedematiens and V. septique were materially attenuated.

The National Medical Research Anaerobe Committee report that in many cases B. perfringens apparently existed as a saphrophyte without etiologic importance. In this same report they come to the conclusion that only two types of anaerobes, namely, B. tetanus and B. botulinus, can be considered to be truly toxogenic. They consider it "doubtful if the products of the other anaerobes can be regarded as toxins in the true sense of the word as their injection is usually followed by immediate toxic symptoms."

Regarding this, I think one might say that our conception of toxin is based on the side chain theory, and that the two chief essentials which must be incorporated into a definition of the word toxin in the restricted bacteriologic sense are:

1. It is a specific poison secreted by a pathogenic micro-organism (specific poison being defined as the substance giving rise to the chief pathologic effects which taken together constitute the disease—Dean.[8]

[7] Vincent et Stodel: Compt. Rend. Acad. Sc., 1917, 164. p. 870.

[8] The Bacteriology of Diphtheria, Nuttal and Smith 1908.

2. This soluble substance secreted in vivo or in vitro is capable by its action on the animal body of the production of a specific antibody.

Other criteria by which toxins may be characterized are: unknown chemical structure, lability, effectiveness of minute dosage, production of pathogenic effects by most of the toxins only after a latent or incubation period.

Dean places 2 and the last criterion in this order as necessary characteristics of a toxin.

B. perfringens filtrate conforms to the definition as suggested and has all the characteristics except that it does not produce pathogenic effects only after a latent period. On this account it would appear to be able to act in a manner different from the toxins produced, for example by B. diphtheriae or B. tetani.

As to which particular manifestations of action and characteristics a poison produced by the growth of a micro-organism must have before it is to be regarded as a toxin in the true bacteriologic sense, is to my mind relatively unimportant to a more accurate understanding of the method of action. It seems an essential conception that the manner in which action is produced must be entirely different if for one there has to be a period of latency, whereas for the other no such period exists, rapidity of action apparently being dependent on the size of the dose, or otherwise expressed on the number of M L D's injected.

For both diphtheria and tetanus toxin the latency period can to some extent be curtailed but not abolished even if many hundred or thousand multiples of the M L D be injected. In the case of diphtheria toxin Dean states this may be reduced to from 8-12 hours.

The same author also states that "the bacilli obtained from cases of diphtheria have as a rule a toxicity which can be expressed by stating that from 0.1 c c to 0.02 c c of the filtrate of a ten days old culture injected subcutaneously into a guinea-pig of 250 gm. weight kills the animal within five days." This size dose is comparable to that of the filtrate of a suitable B. perfringens culture.

No attempt was made to investigate the mechanism by which death was produced by the filtrate of B. perfringens cultures, although such an attempt might throw light not only on B. perfringens toxin but also on the more thoroughly investigated toxins of B. diphtheriae and B. tetani. A classification of toxins based on manner of action would be, I think, a more likely outcome than that the filtrate of B. perfringens cultures would be excluded from the group of true bacteriologic toxins.

Weinberg and Seguin place five anaerobes in the following order of frequency and importance etiologically: (1) B. perfringens, (2) B.

oedematiens, (3) B. sporogenes, (4) B. fallax, and (5) V. septique. B. sporogenes would appear to be the most dangerous of those anaerobes (or aerobes) which are the cause of the putrid odor so frequently noticed in war wounds. V. Septique while not so frequently encountered, has always shown marked pathogenicity, so that antiserum to 1, 2, and 5 at least is desirable and if available should allow specific treatment in the great majority of cases. The antiserum prepared by these authors to both B. oedematiens and V. septique is strictly an antitoxin and was eventually obtained in high potency—about 50,000 or more antitoxic units to 1 c c. Thus one can state that as far as these three strains—B. perfrigens, B. oedematiens and V. septique—are concerned, their pathogenicity is due to an exotoxin against which with suitable strains it is possible to immunize horses and obtain potent antitoxins. It would appear that each antiserum was specific for all members of that strain, but that antitoxins for B. perfringens would not be effective against V. septique, B. oedematiens or vice versa.

As has been shown, it is possible to produce a combined antitetanus and antiperfringens serum from one horse. That anti-B. oedematiens and anti-V. septique could also be combined is possible, though on this I have not noticed any references in the literature at my disposal.

Experimentally all the results point to the great value of the prophylactic use of antiserum specific to the infecting anaerobe, and to the urgency of the earliest institution possible of specific treatment once the disease is recognized. Weinberg and Seguin's controlled cases are encouraging results for the use of specific treatment provided there is as little delay as possible before it is started, and care is taken to determine which anaerobe (or anaerobes) are present.

Judging from the data supplied by the studies of the anaerobes found to be etiologically important in war wounds, it would appear advisable in civilian cases to inject all three antitoxins as early as possible, and to determine the anaerobe (or anaerobes) present in the particular case so that the administration of the appropriate antitoxins could then be pushed.

Weinberg's antitoxin to V. septique and B. oedematiens would appear to contain at the least 10 antitoxic units per cubic centimeter of serum as defined by the United States Public Health Service. One could thus administer daily a minimum of 250 antitoxic units to all three anaerobes in a total of 75 c c of serum until the etiologic agents for the particular case were determined.

As pointed out by Van Beuren, the specific treatment should be regarded as an adjunct to full and proper surgical measures, and should not in any way supplant these surgical steps.

INFECTIOUS ABORTION OF SWINE

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Although the subject of infectious abortion of cattle has been exhaustively studied for the past 35 years, it is apparent that little intensive research has been directed toward the same disease of swine. Records in our laboratory indicate either that the importance of the loss due to abortion of swine is becoming more appreciated, or that there is an alarming increase in its prevalence. A search of the literature reveals one paper on the subject. Good and Smith [1] studied three field outbreaks and report the causative agent to be B. abortus (Bang), which they succeeded in isolating from an aborted fetus.

For the past five years a portion of the routine work of this laboratory has consisted of making agglutination tests of blood from cattle suspected of infection with abortion disease. Occasionally, blood samples from swine have been received for the same test, and these were tested with the same cattle strain antigen. The results of these tests are recorded here, with a brief history of the herd when available.

1. Blood from a sow: According to the history of the herd, 9 of 15 sows aborted in the summer of 1917, and 6 of 10 in the summer of 1918. The agglutination test of the one sow was negative.

2. Thirty-nine blood samples from a herd of 200 brood sows: Seventy abortions had occurred within 6 weeks. The results of this test were startling; twenty-eight (66%) showed agglutination in a serum dilution of 1 : 100; eight (21%), including one boar, showed no trace of agglutination, and the other five agglutinated only in dilutions lower than 1 : 100.

3. Six samples from a herd with a general history of abortion: Two gave complete agglutination with a dilution of 1 : 500; the other 4 were negative.

4. Three samples from a small herd: Four sows, which had been bred to an old boar, had aborted. Nine young sows, which were raised on the same farm, were bred to a young boar and 8 aborted. The 4 old sows were bred to the young boar, and again all 4 aborted. Six sows were bought after having been bred; they were brought to this farm and placed in the same pens; 1 aborted. Of the 3 blood samples, 1 was negative; another agglutinated in a dilution of 1 : 500, and the blood from the old boar was negative.

5. One sample from a sow which had recently aborted; another from a boar: Both samples agglutinated completely in a dilution of 1 : 500.

6. Eighteen samples from a herd of 28: Four abortions had just occurred in this herd. Of these samples, 9 agglutinated in a dilution of 1 : 100 or higher;

[1] Jour. Bacteriol., 1916, 1. p. 415.

9 were negative. Of the 9 positive reactions, 2 had aborted, 1 was said never to have been bred, and 2 had been bred and were presumably pregnant at the time of bleeding. The record on the remainder of the herd was incomplete.

2b. Samples from 7 brood sows from the same source as herd 2a. Four of these samples agglutinated in a dilution of 1 : 100; 3 were entirely negative.

7. Fourteen samples were tested from a herd; no data were available. Twelve agglutinated in a dilution of 1 : 100 or higher; only 2 were negative.

2c. Blood from 6 gilts suspected of infection from the same source as herds 2a and 2b: Two samples agglutinated at 1 : 200; 2 agglutinated at less than 1 : 100; and 2 were entirely negative.

8. Five samples were tested from a small herd with a general history of abortion. Three agglutinated at 1.200; one was positive only at 1 : 50; the other one only at 1 : 20.

9. Recently a sample from a valuable brood sow was tested. This sow showed a vaginal discharge, and had been bred without conceiving. Agglutination was complete at a dilution of 1 : 500.

10. Twelve cholera hyperimmune hogs, both male and female, which may be regarded normal as to history of abortion, were tested. Five showed only a doubtful trace of agglutination in dilution of 1 : 20; one showed a doubtful trace at 1 : 50; all others were negative.

These brief histories cover a report of tests of 114 hogs of both sexes, and of varying ages, with these results:

Results of Tests

		Positive at 1: 100 Dilution	Negative at 1: 100 Dilution
Sows	92	57	35
Boars	3	1	2
Gilts	7	3	4
Normal hogs	12	0	12
	114	61 (53%)	53 (46%)

From these data it appears that 50% of swine of mixed origin, or 62% of animals from herds with a history of infectious abortion, gave positive agglutination in a dilution of the serum regarded as diagnostic of this disease of cattle. Furthermore, the antigen used was in all cases a mixed suspension of two or three strains of B. abortus (Bang) of cattle origin. The significance of these results is apparent, and at the earliest opportunity attempts were made to isolate this organism from swine.

In July, 1919, a portion of afterbirth from a sow of herd 2a was received. The tissue was triturated in normal salt solution, and 1.5 c.c. of the washing was injected intraperitoneally into each of two guinea-pigs. At the same time these animals were bled and the serum tested with B. abortus (Bang) cattle strain antigen; no agglutination appeared in any dilution. Four weeks after inoculation the pigs were bled and the blood of both pigs agglutinated the antigen completely at 1 :500.

Five weeks after inoculation the guinea-pigs showed considerable emaciation and were killed. There were numerous whitish nodules in the liver; much enlarged spleen; some nodules in the lungs, and a large quantity of serous fluid in the pleural cavity. Serum-agar slants and shakes were seeded from the liver and spleen. No growth was perceptible after 48 hours, but on the third day all shakes and slants showed a moderate surface growth of an apparently pure culture. Suspensions of this growth were tested with a known positive cow serum, and were completely agglutinated in all dilutions up to 1 : 500.

Rabbits were injected intravenously with suspensions of killed organisms of four strains of B. abortus (Bang) of cattle origin, and of this strain of swine origin. Potent antiserum was developed against the respective homologous antigens. Cross-agglutination tests between all cattle strains and the swine strain showed the same degree of agglutination as with homologous antigens. No distinction could be made between the cattle and swine strains.

Antiserum against the swine strain was absorbed by antigen of each of the four cattle strains, and antiserum against each of the four cattle strains was absorbed by antigen of the swine strain. Agglutinins were absorbed completely in all cases, as indicated by test of the absorbed serum with the respective homologous antigen.

Gram stains from an agar slant culture of the swine strain showed the organism to be gram-negative, and to conform to the well-known morphology of typical B. abortus (Bang). No motility was perceptible, and flagella could not be demonstrated.

Comparative cultural tests were made simultaneously with the four cattle strains and the one swine strain. All five strains conformed to the published characteristics of B. abortus (Bang), except for a few slight variations. Several additional characteristics are worthy of note:

When inoculated into slightly alkaline 2% glucose agar shakes the 4 cattle strains first showed the usual subsurface growth to a depth of about 3 mm. At the lower limit of this growth a pronounced zone of growth produced a distinct "diaphragm." By the third day the surface growth was fairly prolific. In the same medium the swine strain showed a few surface colonies at 24 hours. and a trace of subsurface growth, but no evidence of the diaphragm.

In 2% glucose agar shake cultures titrated to slight pink to the Andrade indicator, and to which 1% of this indicator was added, the color was reduced at 24 hours to the depth of growth, below which the color remained sharply defined for 3 days. This decolorization progressed slowly so that at 7 days the color had entirely disappeared. All strains behaved alike in this respect.

A perceptible surface blackening was developed by all strains on lead acetate agar at 24 and 48 hours. This darkening did not approach the degree produced by B. paratyphosus B on the same medium, but had more the appearance of a grayish pigment; no such color appeared on plain agar.

Serum-water was prepared with the addition of 1% of the Andrade indicator and 1 per cent. of the following sugars and fermentable substances: dextrose, lactose, saccharose, mannite, arabinose, xylose, dulcite, inosite, maltose, levulose, galactose salicin and dextrin. Moderate acid was produced by all four cattle strains in arabinose; a doubtful trace of acid was produced in the same medium by the swine strain and sharp differentiation was produced on repetition. Three cattle strains produced a trace of acid in levulose; no acid was perceptible in the same medium inoculated with the fourth cattle strain nor with the swine strain.

Except for the reaction in arabinose serum-water, none of the slight cultural variations were sufficiently distinctive to counteract the evidence of close rela-

tionship, if not the identity of the five strains, as indicated by direct and cross-agglutination, and by absorption tests.

Artificial infection of guinea-pigs in effecting the isolation of the swine strain, together with development of lesions and agglutinins, and cross-agglutination with cow serum and cattle strain antigen, appears to establish the identity of this swine strain with typical B. abortus (Bang).

SUMMARY

The history of eight herds indicates that infectious abortion of swine is a serious and increasingly important source of loss to the swine industry.

Inoculation of two guinea-pigs with triturated afterbirth from an aborting sow resulted in an infection, as demonstrated by the development of agglutinins and typical lesions. At the time of inoculation the blood of the two guinea-pigs did not agglutinate a B. abortus (Bang) antigen. Four weeks after inoculation the blood agglutinated this antigen completely in all dilutions up to and including 1 : 500.

An organism was isolated from the liver and spleen of both guinea-pigs, which, except for slight cultural peculiarities, is indistinguishable morphologically, culturally, or agglutinatively from typical B. abortus (Bang).

Rabbit antiserum for four cattle strains and for this swine strain gave cross-agglutination to the same titer as with the homologous antigens.

Antigen of the swine strain completely absorbed agglutinins from antiserum for each of the four cattle strains.

Antigen of the four cattle strains each completely absorbed agglutinins from antiserum for the swine strain.

EFFECT OF CARBOHYDRATE ON AMINO ACID UTILIZATION OF CERTAIN BACTERIA

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That a utilizable carbohydrate influences the changes in protein and amino-acids in bacterial cultures, is so well known as to require no comment. The phenomenon has assumed undue prominence in the literature because various investigators do not agree in their interpretations. The two outstanding features of bacterial metabolism, which have been emphasized by Kendall, Walker and Day[1] in a series of experiments extending over several years, are: (1) Protein in the presence of a fermentable sugar is spared. (2) When sugar is present, protein metabolism is reduced to the minimum required for structural purposes. Herman and Rettger,[2] however, using a simple buffered medium in their series of experiments, came to the conclusion that "Kendall and Walker's conception that the presence of glucose delays the production of the proteolytic enzyme cannot be accepted. In the tests in which the buffer reagent was employed the proteolytic enzyme appeared as soon in the sugar media as in the plain bouillon." They ran parallel tests on mediums containing 0.2% and 0.4% dextrose, with 0.25% beef extract, 0.5% peptone, and 0.5% NaCl, one series without, and the other series with 0.5% K_2HPO_4. By following the changes by tests on successive days, an interesting sequence, when properly interpreted, is recorded in their table. Why they would limit the concentration of sugar to any percentage is not clear. The action of the buffer in their experiments is clearly to hold the concentration of hydrogen-ion below the toxic limit, and thus enable the organisms to exhaust the last trace of such small quantities of sugar. The sugar having been exhausted, the organisms of course then turn to the amino acids for their energy needs, which accounts for the sequence of changes shown in their table.

They conclude that "the presence of sufficient buffer in a medium encourages continued normal nitrogen metabolism." In other words, they argue for keeping the hydrogen-ion concentration nearer neutral-

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[1] Jour. Am. Chem. Soc., 1913, 135, p. 1201.
[2] J. Bacteriol., 3, 389, 1918.

ity. In compliance with this requirement, I have used 4% dextrose, with normal sodium hydroxid for titrating the acid as rapidly as formed with brom-cresol-purple in the culture as an indicator. After 12 hours the culture had to be watched continuously because of the rapid formation of acid, but after a period of 36 hours of this marked activity, the reaction became stationary. Although still maintained at the neutral point (P_H 7.5, by the hydrogen electrode) and kept for a period of 32 days, no evidence of "continued normal nitrogen metabolism" could be detected. The proteolytic enzyme had not appeared, indol was negative, ammonia formation was not greater than in the untitrated control, and sugar was still present.

Aside from the interesting fact that the activity of a culture can be arrested by the accumulation of products of its metabolism other than the toxic ion of hydrogen, perhaps in this instance the lactate ion principally, it is seen that even under the most favorable conditions of hydrogen-ion concentration, i. e., P_H 7.5, amino acid utilization for energy needs is definitely inhibited as long as sugar is available.

The paradoxic result of finding sugar in the phosphate medium but not in the 0.2% dextrose phosphate-free medium, requires explanation. Why was it not present in the unbuffered medium? That such a small trace of sugar was detected in the presence of 0.5% phosphate is most unusual. In regard to this, Berman and Rettger insist that "these results were indeed unexpected, and the tests were repeated, with identical results."

Whether sugar was present or absent, therefore, decides the interpretation of what follows. Here is the crux of the whole matter. The following points are offered as evidence against the claim that sugar was present: (1) It should be remembered that there is a common practice among bacteriologists of rendering beef infusion sugar free by incubating for 24 hours with dextrose fermenting organisms. How is such a practice to be justified if there is a "residuary carbohydrate" left in the culture even after 27 days' incubation? (2) To test the correctness of these findings one has only to kill such a culture, divide it into two portions, add at least 0.1% of sugar to one portion, and test both portions with Benedict's solution to discover that even with this extra 0.1% of sugar added to the "residuary carbohydrate" spoken of by these authors, the precipitate of cupric phosphate is so heavy in both tubes as to obscure entirely any positive sugar reaction. Even after repeated boiling and standing for 24 hours the tubes differ in no respect in appearance from an absolutely sugar free

control. (3) When phosphate in such a concentration is known to be present, failure to remove it from the solution before testing for small traces of sugar, would be considered a serious oversight by any one acquainted with the limitations of Benedict's solution as a reagent for sugar in the presence of certain other substances.

Berman and Rettger claim that "fermentable sugars in moderate amounts do not affect the nitrogen metabolism of bacteria—under conditions of favorable environment," and that "the common belief in a so-called 'sparing action' of sugar in a protein medium is untenable in the light of these experiments. According to this idea protein is spared from all participation in the metabolism." I am unable to find anywhere in the reports of the work which they refute, any statement that "protein is spared from all participation in the metabolism," under any conditions. Growth requirements imply participation of protein in the metabolism.

The disagreement in our interpretations throughout probably originates from the experiment in which they found "a residuary carbohydrate" by Benedict's method, even in the presence of 0.5% phosphate, after 27 days' incubation, and though only 0.2% carbohydrate was present in the beginning. According to my experiments, the limiting hydrogen-ion concentration of B. proteus i. e., P_H 4.8, is sufficiently high to allow complete removal of 0.2% dextrose in 36 hours even in the usual (unbuffered) medium. Their table shows practically the same result in the 0.2% dextrose unbuffered medium, namely, positive sugar test up to 24 hours, but negative after 3 days. But why should it persist in the buffered medium? Phosphate should facilitate its removal rather than give rise to a "residuary carbohydrate."

Further disagreement arises from the fact that they do not make clear the distinction between protein hydrolysis and amino-acid utilization. These must be specified by more definite terms than the inclusive term "protein metabolism." Both should not be included when only one is meant. When the one occurs we see liquefaction without putrefaction; when the other occurs we find the formation of indol, H_2S and the genuine putrefactive changes characteristic of amino acid disintegration. They usually occur together but not necessarily. For example, many organisms which do not liquefy plain gelatin will produce a decided softening (acid hydrolysis) if a utilizable sugar is present. In other words, in the sugar-free gelatin cultures of,

say, B. coli, putrefaction but not liquefaction occurs; in the sugar gelatin, liquefaction but not putrefaction, showing that either phase of "protein metabolism" may occur alone. To demonstrate this softening of gelatin by acid hydrolysis, one has only to acidify a tube of sterile gelatin medium to P_H of about 4.5, with some acid, e.g., lactic, and compare its consistency after a few days at 37 C. with a similar control tube to which has been added a proportionate quantity of sterile water. Since the medium is kept sterile, this effect on the gelatin certainly is not one of "protein metabolism" and yet this softening of gelatin has often been erroneously offered as evidence that protein metabolism occurs in the presence of sugar.

It is not impossible that certain organisms have been or will be found which could utilize both amino acids and sugars at the same time, but such strains will be the rarest exception to the rule, and their occurrence would not in any sense invalidate the general proposition set forth in the work of Kendall, Walker and Day as a valuable working hypothesis and basis of interpretation in metabolic studies.

SUMMARY

A culture of B. proteus containing sufficient carbohydrate shows no evidence of amino acid utilization, even though the reaction of the culture was maintained at neutrality during its entire period of active growth, and for an additional period of one month following cessation of activity.

The softening of gelatin occurring in sugar-gelatin medium is an acid rather than an enzymic-hydrolysis, and should not be interpreted as a part of protein metabolism.

A COMPARISON OF THE MORPHOLOGIC, CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF B. ABORTUS AND B. MELITENSIS *

STUDIES ON THE GENUS BRUCELLA NOV. GEN. I

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The problem dealing with the possible pathogenicity of B. abortus (Bang) to human subjects investigated in the last ten years from various points of view was placed in a new light when, in 1918, Alice E. Evans [1] demonstrated by morphologic, biochemical and serologic studies a close relationship between the organism responsible for infectious abortion of domesticated animals and the so-called Micrococcus melitensis, the cause of the well-known undulant or Malta, or Mediterranean fever in man. Moreover, the peculiar latency in tissues and the apparent ubero- and sexotropic character of the two organisms in cattle and goats, respectively, lend additional support to the above contention. To the bacteriologist, however, who obtains his information mainly from the meager descriptions and accounts given in the usual textbooks instead of from a comparative study of authentic cultures in vitro and in vivo, this correlation of facts appears impossible. We mention in this connection the conservative attitude of a number of English bacteriologists, who place the causative organism of Malta fever with the coccus group and fail to recognize the repeated observation that this organism may appear in smears made from young cultures and even from tissue material as a typical short rod. On the other hand, the small microbes found in some forms of infectious abortion have, since the classic studies of Bang and Stribolt,[2] been accepted as distinct rods which, however, may occasionally appear in exudates as a "coccobacillus." Furthermore, an analysis of the descriptions dealing with the cultural and biochemical characteristics of the two organisms under consideration reveals only differences of minor importance and adds considerable evidence to the conception of a close relationship of B.

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* The letter B. is used in this series of papers for the suggested genus Brucella Nov. gen. and enhances the suggestions made by A. E. Evans (Footnote 1).

[1] Jour. Infect. Dis., 1918, 22, p. 580.

[2] Ztschr. f. Thier. med., 1897, 1, p. 241.

abortus and "Micrococcus" melitensis. Irrespective of the fact, that Alice E. Evans supported her conclusions by the presentation of observations on several strains of the two types of bacteria, a number of bacteriologists have expressed to us their inability to accept this new conception. This in part may be due to the unfortunate mistake which Miss Evans committed in correlating B. bronchisepticus with B. abortus and with "Micrococcus" melitensis. As is well known, B. bronchisepticus is a motile, strongly alkali-producing rod, which is either related to the pyocyaneus group (Smith),[3] or to B. pertussis (Ferry and Noble).[4]

In order to verify the various statements and preparatory to a number of experiments to be considered in this series of papers, we studied the morphology and biochemical reactions of 21 cultures, which had been identified by various authoritative laboratories in the United States, England, Algiers and Italy as "Micrococcus" melitensis. We included in this comparative study 32 cultures of B. abortus isolated in this country or in England from aborted fetuses or pathologic discharges, or milk of cattle and hogs.

All the cultures were repeatedly plated on glycerol-peptic digest agar and are kept in triplicate sets on the same medium at room temperature. The tests to be recorded have been repeated at least three times, all the strains being tested for the most part simultaneously. A selected number of strains used in the serologic tests reported in the second paper were studied more extensively after rapid transplantation on the same medium for at least 10 to 15 generations. The inoculated tubes were kept sealed with paraffin wax.

MORPHOLOGY

"Micrococcus" melitensis.—The stock cultures designated "Micrococcus" melitensis, when grown on peptic digest agar or broth with a reaction of P_H 7.2-7.4 for 24 to 36 hours at 37 C., revealed in preparations stained with gentian violet short, stumpy, oval or egg-shaped rods frequently tapered at both ends. Identical smears stained by Gram's method and counter-stained with dilute carbol-fuchsin furnished pictures in which the organisms appeared more coccoid in morphology. This observation is quite in accordance with the findings of Fabyean,[5] made on B. abortus; he noted that carbol-fuchsin accentuated the diameter and gentian violet, the length. In hanging drop preparations the organisms are immotile, noncapsulated and appear

[3] Jour. Med. Res., 1913, 29, p. 299.
[4] Jour. Bacteriol., 1918, 3, p. 193.
[5] Jour. Med. Res., 1912, 26, p. 477.

more like elongated cocci or diplococci. Frequently in the water of condensation or liquid mediums, short chains consisting of from 4 to 10 single, elongated, influenza-like bacillary or stumpy, coccoid elements can be recognized. Strain 2 produces these forms rather frequently; while strains 1, 5, 7, 18, 22 and 26 form single coccoid rods, which are evenly distributed in the stained preparations. Strains 8, 9, 20, 21 and 23 invariably appeared in young cultures as fine small rods in parallel grouping. The individual elements may stain more intensely at both ends and measure from 0.8-1.8 microns in length and from 0.4-0.6 microns in width. The forms most frequently recognized in young cultures on glycerin peptic digest agar, are illustrated in microphotographs 1-6.

It is quite evident that we are unable to recognize the interpretation of Eyre,[6] who considers these bacillary forms to be staining artefacts. And again the finding of bacilli in 24 hour old cultures on the most suitable mediums with an optimum reaction and oxygen refutes the conception that they are involution forms. We admit, however, the occasional occurrence of a cultural growth after 12 to 18 hours' incubation on suitable solid substratums, which in carbol-fuchsin or thionin preparations consists mainly of coccoid-like elements, indistinguishable from the elements of a young culture of meningococci. A few incomplete tests suggest that definite cyclical changes in the development similar to those described for a variety of organisms by Clark and Ruehl [7] exist also for the B. melitensis. A detailed study of this phase of the problem is in progress.

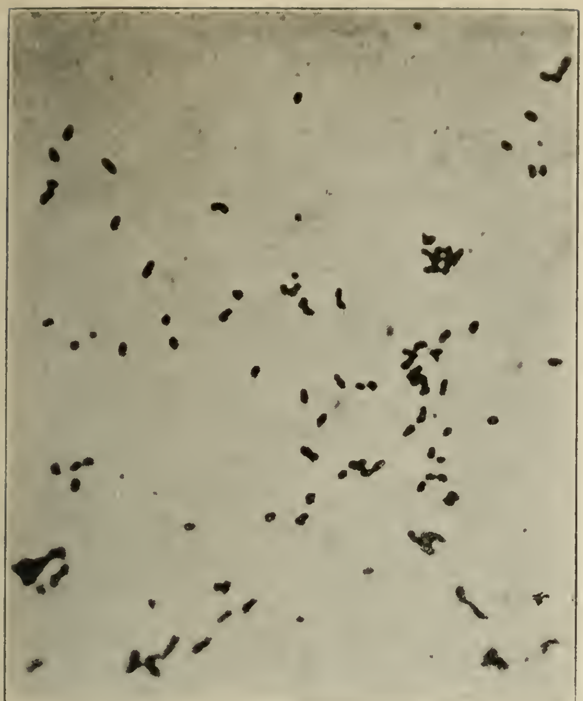
When stained in thin preparations the organisms of all our strains are gram negative. Repeated tests failed to demonstrate flagella by the method of von Ermengen. Our observations on the morphology of "Micrococcus" melitensis support, therefore, the finding of Durham,[8] Galli-Valerio,[9] Besson,[10] Pollaci,[11] and Muir and Ritchie.[12] We therefore concur in the interpretation given by Miss Evans and demand that the generic name "Bacterium" be given to the causative organism of undulant or Malta fever.

[6] Kolle and Wassermann's Handbuch d. pathog. Microorg., 1913, 4, p. 424.
[7] Jour. Bacteriol. 1919, 4, p. 615.
[8] Jour. Path. & Bacteriol., 1899, 5, p. 377.
[9] Centralbl. f. Bakteriol., I, O, 1904, 35, p. 81.
[10] Practical Bacteriology, London, 1913, p. 475.
[11] Centralbl. f. Bakteriol., I, Ref. 1908, 42, p. 676.
[12] Manual of Bacteriology, 7th Ed., London, 1919, p. 501.

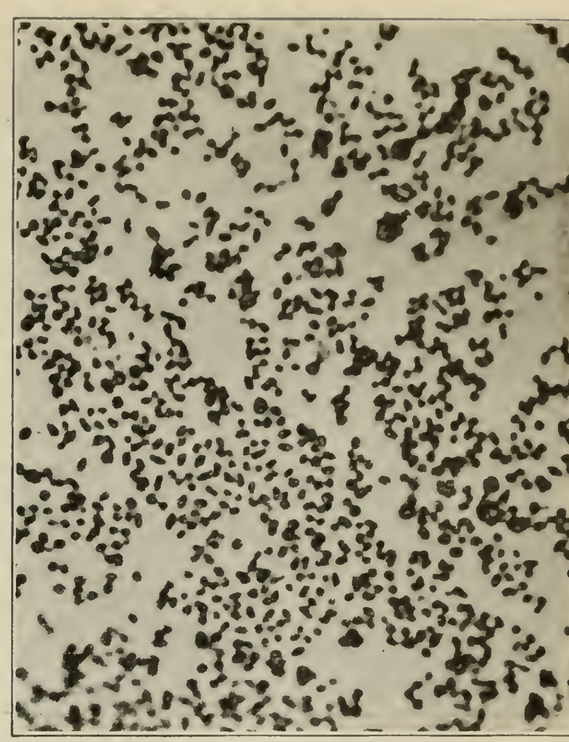
B. abortus.—The morphologic appearance of the various strains of B. abortus on the same medium are similar to those of B. melitensis. Again, in preparations stained with gentian violet short ovoid or longer rods are demonstrated. The diphtheroid-bacilli-like grouping of the small rods and the indications of granular staining are perhaps more frequently seen in young B. abortus cultures, than in those of B. melitensis. The length varies between 0.4-2.2 microns and the width between 0.4-0.8 micron. Short chains of coccoid elements are also noted in the water of condensation of young cultures. Recently isolated strains, which are not fully adapted to the new oxygen requirements and the new substratum, appear more coccoid than old, vigorously growing stock cultures. The organisms are always distinctly gram-negative. Microphotographs 7 and 8 illustrate these observations fully (see also, Figs. 1 and 2 on Tafel II, Arb. a. d. k. Gsundhtsamte., 1912, 43, p. 129, and Kolle-Wassermann's Handb. d. pathog. Microog., 1913, 6, p. 299).

In this connection it may appear advisable to recall briefly the various statements relative to the morphologic appearance and the botanical classification of B. abortus published in the literature. Preisz[13] placed the causative organism isolated by him from cases of infectious abortion on account of its irregular staining reaction and its diphtheroid-like grouping with the corynebacteria. It is, however, not unlikely that the organism described by Preisz is not identical with the bacillus of Bang.[14] According to Novak,[15] B. abortus resembles the coccobacillus of chicken cholera, and it is therefore grouped with the pasteurella or hemorrhagic septicemia bacilli. Holth[16] considers the organism on "ausgesprochener Kokkobazillus," and Zwick and Zeller[17] noted several strains which possessed a "fast kokken-ähnlices Aussehen," which in turn resembled by darkfield illumination the bipolar bacteria of fowl cholera or swine plague. Fabyean[18] states that "there is some variation in length which in some individuals may be equalled by the diameter, this type suggests a coccus." Our personal observations are therefore fully corroborated by the findings made by other workers. We found it impossible to distinguish B. melitensis from B. abortus when using cultures with fictitious labels prepared from our stock sets, irrespective of the fact that our constant working with the strains should have impressed on our mind the essential differentiating characteristics. On morphologic grounds the organisms of undulant fever and of infectious abortion of domesticated animals must therefore be considered as identical and must be placed together in the genus bacterium. For reasons to be given in detail in the second paper it is proposed in accordance with the suggestions made by Buchanan[19] of the Committee on Classifications of the Society of American Bacteriologists, that a genus,

[13] Centralbl. f. Bakteriol., I, O, 1903, 33, p. 190.
[14] Zwick and Zeller, Footnote 17, p. 5.
[15] Ann. de l'Inst. Pasteur, 1908, *22*, p. 541.
[16] Ztschr. f. Infektionskrankh. parasitärkrankh. u. Hyg. d. Haustiere, 1911, 10, p. 208.
[17] Arb. a. d. Gsundhtsamte, 1912, 43, p. 11.
[18] Jour. Med. Res., 1912, *26*, p. 476.
[19] Abstracts Bacteriol., 1918, *2*, p. 8.

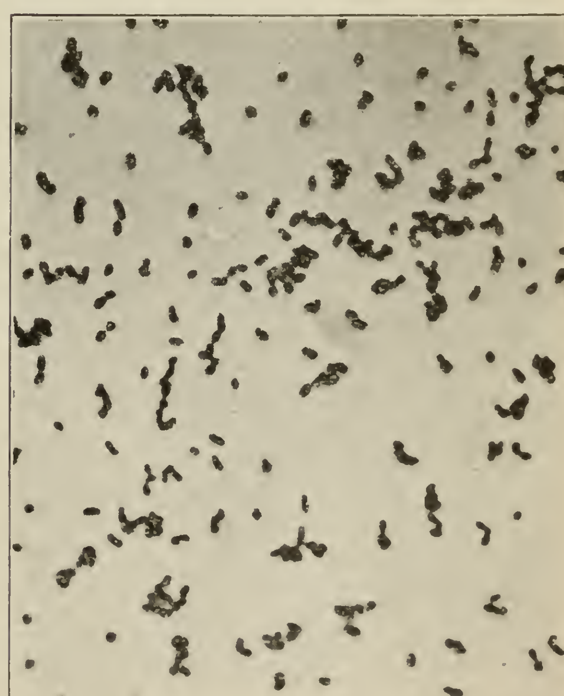


Strain 7.



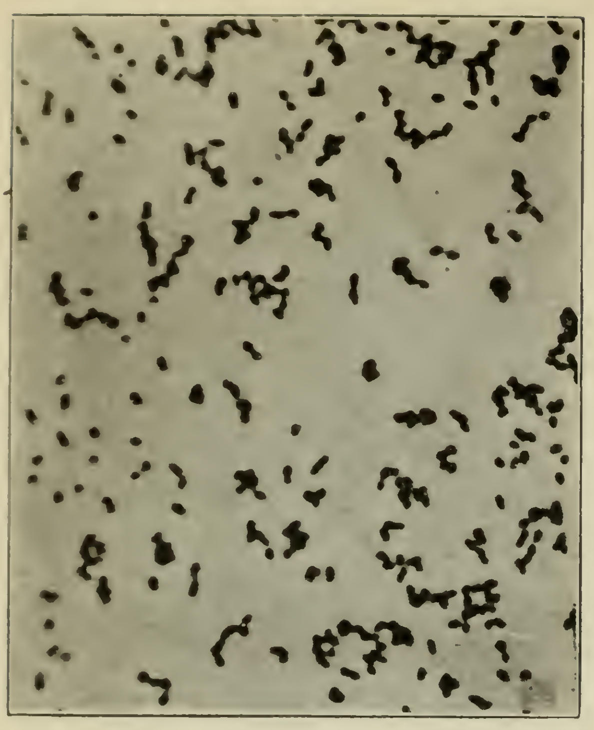
Strain 1.

Strain 22.



Strain 18.

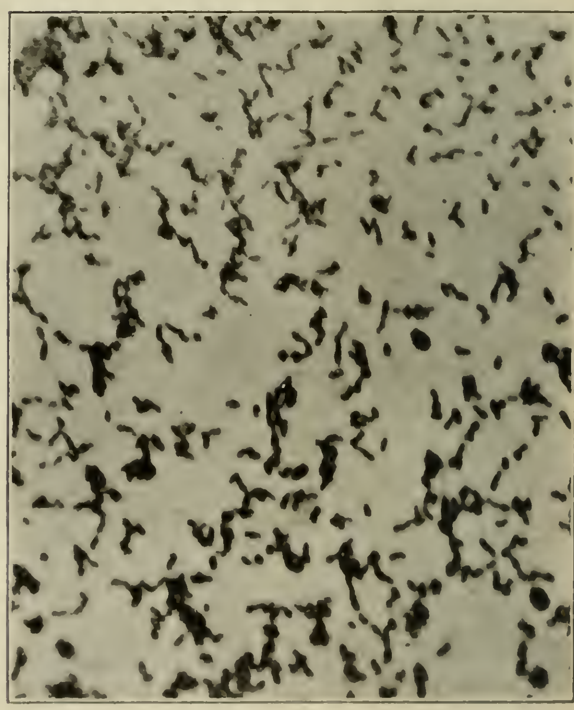
Fig. 1.—B. melitensis, × 1,500.



B. melitensis strain 26.

B. melitensis 20.

B. abortus strain 80



B. abortus 12.

Fig. 2. B. melitensis and B. abortus; × 1,500.

for which we propose the name "Brucella," be created in the family Bacteriaceae to separate and to distinguish properly these important pathogenic micro-organisms from the other members of the recognized genus bacterium, which is already overburdened with representatives, which have little in common with the B. melitensis and B. abortus.

CULTURAL CHARACTERISTICS

The descriptions given by Horrocks,[20] Eyre,[6] and others for the growth of B. melitensis and by Bang and Stribolt,[2] Holth,[16] Zwick and Zeller,[17] and Fabyean,[5] for that of B. abortus on various solid and liquid mediums are fully in accord with our own observations, and it is unnecessary to duplicate the recital of facts. We intend to point out chiefly the slight differences which can be noted and perhaps be chosen to separate on cultural grounds the B. melitensis from the B. abortus.

It is well known that both organisms grow slowly, even on suitable medium visible growth is not recorded before 36-48 hours' incubation at 37 C. It is furthemore established that primary isolation of B. abortus from pathologic material on glycerol or serum agar can only be accomplished successfully by either reducing (Bang and Stribolt, Novak and also Fabyean) or increasing (Holth) the oxygen tension of the atmosphere, in which the organism is placed for artificial cultivation. A similar adaptation for B. melitensis has not been reported in the literature. It is not unlikely that the usual isolation of this organism from the blood stream of patients in liquid medium or the milk of goats explains the difference. The peculiar adaptation phenomenon of B. abortus to varying oxygen tensions, when first isolated from amniotic fluid or uterine material in general is probably merely the result of its intra-uterine existence as clearly demonstrated by McFadyean and Stockman [21] and also by Holth. Through the observations of Schroeder and Cotton,[22] A. S. Evans [23] and Steck,[24] who isolated the B. abortus directly from milk in ordinary petri-dishes, even in gelatin medium, and the fact that even prolonged sojourn of the organisms in guinea-pig tissues does not confer this adaptation phenomenon again, it is clearly indicated that the adjustment to definite oxygen tensions is primarily a characteristic for the organism living in the uterine cavity. As we had no access to actual cases of undulant

[20] Report of the Commission on Mediterranean Fever, London, 1905, Part 1, p. 5.

[21] Report of Departmental Committee on Epizootic Abortion, Append. to Part 1, Lond. 1909, p. 25.

[22] Twenty-Eighth Annual Report of the Bureau of Animal Industry, Dept. of Agricult. 1911, p. 139.

[23] Jour. Infect. Dis., 1916, 18, p. 437.

[24] Schweizer Arch. f. Tierheilk., 1918, 60, p. 547.

fever, we are unable to express an opinion concerning the adaptation of B. melitensis to varying oxygen tensions. We noted, however, repeatedly that cultures made from guinea-pig spleens, which had been injected with various B. melitensis strains 3 to 4 months previous to the time of necropsy, gave only growth in paraffin sealed blood or glycerin peptic digest agar tubes. Plain agar plates or veal infusion agar slants loosely closed with cotton plugs frequently remained sterile. Some tests indicated that the composition of the medium influences considerably the chances of the primary isolation of B. abortus and B. melitensis. Our pig or beef-liver peptic digest agar [25] is admirably suited for this work. Dr. J. Traum of the University of California isolated on this medium in open unsealed slants B. abortus directly from the amniotic fluid in the stomach and from the liver of aborted pig's fetuses. The use of "nutrose," "somatose" and beef serum by English experts on Malta fever is repeatedly recorded, and it is not unlikely that these "growth accessory" substances helped in the primary isolation of B. melitensis and acted in a similar manner to our digest agar. The majority of our strains were naturally stock cultures, grew therefore abundantly on all culture mediums and appeared fully accustomed to saprophytic life. The three paramelitensis strains 9, 22 and 23 and strain 18 were shy growers when received and even repeated subculturing only slightly enhanced the cultural vigor in comparison with the other strains. We found the optimum reaction of the medium to be a H-ion concentration of P_H 7.2-7.4. Both types of organisms are slightly more alkali than acid tolerant.

The growth on glycerol peptic digest agar or hormone blood-agar plates appears at 37 C. in form of small convex, glistening, pearly-white, droplet-like colonies, which may develop into colonies of from 2-8 millimeters in diameter. The paramelitensis strains 22 and 23 produced sometimes rather granular, dull, comparatively flat colonies; this phenomenon was particularly marked on dry plates. B. abortus colonies cannot be distinguished from those of B. melitensis.

On agar slants a fine granular film appears in from 24-36 hours; after 3 days a slight brownish tinge changes the moist, well defined growth. Development continues on peptic digest agar for weeks, even at room temperature, until a stringy, greasy, rather thick layer covers the inoculated agar surface. This growth remains amber or honey-like yellow or perhaps caramel-like brownish (see Fig. 1, Plate 28, twenty-

[25] Stickel and Meyer, Jour. Infect. Dis., 1918, 23, p. 68.

eighth annual report of the Bureau A, U. S. Dept. Agri.), for B. abortus strains even after six weeks' incubation. B. melitensis strains 1, 2, 3, 5, 7, 8, 19, 20, 21, 23 and 24, however, changed their growth to a deep chocolate or dirty chestnut brown, some even to a dull ebony black. This pigmentation of the bacterial layer is usually more marked at the upper portion of the slant and may be accompanied by a slight or pronounced darkening of the agar substratum. Intensive dark pigmentation is regularly observed with the cultures of B. melitensis mentioned and differentiates these strains distinctly from all our B. abortus strains. The time of incubation to produce this pigmentation is not constant and may vary from 8 to 30 days. It is, however, emphasized that a number of B. melitensis cultures (4, 6, 9, 10, 11, 18, 25, and 27), which must be classified serologically as typical strains, have failed to produce a darker pigment than on B. abortus cultures and differ therefore in no respect from the latter. Crystals, which are probably due to the increasing alkalinity of the medium, were observed only after two weeks' incubation in our digest agar mediums. In veal infusion agar they may appear on the sixth to tenth day of incubation. Agar shake cultures of all strains fail to show a zone of growth as mentioned by a number of writers; there is a thick growth on the surface, which may also extend slightly beneath the surface.

In gelatin, all our strongly pigment-producing B. melitensis strains developed dark brownish granular colonies after incubation of from 10 to 30 days. The B. abortus strains acted similarly. The medium was never liquefied.

In veal infusion or digest broth a slight initial turbidity, which is followed by a gradual clearing and by a stringy, tenacious sediment, occasionally with a slight pellicle or ring formation, was noted for all the strains studied after 5 to 10 days' incubation. Our B. melitensis strains 9, 18, 22, 33, and B. abortus cultures 5, 12, 13, and 19 produced a scaly, powdery sediment with little or no turbidity of the supernatant broth medium.

Cultures on potatoes may give varying results, depending on the age of the tuber and its reaction. On properly chosen, slightly alkaline, moist potato-cylinders the behavior of the majority of our B. melitensis strains is in some respect characteristic. Inoculated from a broth culture or the water of condensation, the visible growth was always distinctly amber yellowish or even brownish after 5 to 6 days' incubation. The following strains behaved in this manner: 1, 2, 3, 4, 5, 6, 7,

9, 10, 11, 19, 20, 21, 22, 23, 24, 25, 26 and 27. B. abortus strains, however, cultivated simultaneously on the same medium and in the same manner, showed only a faintly yellowish hue. After 3 to 4 weeks of incubation, they may show the well-known glanders bacillus-like appearance (McFadyean and Stockman[26]). At this period the B. melitensis strains mentioned are already deep brownish. Very old melitensis cultures demonstrate a more intense pigmentation of the bacterial growth and marked brownish discoloration of the potato itself in contrast to the generally light coloring of that of B. abortus. Variations in the shading of the color among the latter strains are not uncommon and again the B. melitensis cultures 8 and 18 behaved, when repeatedly tested on potatoes, like the B. abortus strains.

Bromcresol purple goat's milk in fermentation tubes is turned slightly alkaline after 5 to 10 days' incubation at 37 C. in the open arm by all the strains tested. The H-ion concentration decreases from P_H 6.6-7.2-7.4. Litmus milk remains unchanged or turns slightly deeper blue in the open arm. Fresh goat's milk with a layer of cream and bromcresol purple as an indicator shows no visible changes even when incubated for three months. In goat's milk litmus whey, the titerable alkalinity of both the B. melitensis and B. abortus strains varies after 10 days' incubation between 0.2 and 0.5 per cent. of a normal HCl solution. The differences in the final reaction are merely the result of differences in the rate of multiplication of the various strains. Poorly growing B. melitensis and B. abortus strains produce a small amount of alkali. The absence of changes recorded in the sterile goat's milk stratified with the cream suggests that the alkaline reaction is caused primarily by the oxidation of the salts of citric acid to alkaline carbonates as recently discussed by Ayers and Rupp.[27]

BIOCHEMICAL REACTIONS

Hiss' serum-peptone-pheno-sulphonephthalein-water, containing 1 per cent. of levulose, galactose, maltose, saccharose, raffinose, mannite, dulcite or inulin are not fermented by the representatives of the genus "Brucella." In glucose and lactose-peptone-phosphate-broth an alkaline reaction develops after 5 to 20 days' incubation at 37 C. The H-ion concentration decreases from P_H 6.8 to 7.6 and to 7.8. This reduction, already observed by Eyre and enhanced by Evans, is con-

[26] Report of Departmental Committee on Epizootic Abortion, Append. to Part I, London, 1909, p. 4.

[27] Jour. Infec. Dis., 1918, 23, p. 188.

stant for all our strains, when final determinations are made after the twentieth day of incubation. Vigorously growing strains as a rule produce this alkaline reaction in a shorter time interval than the poorly growing types (for example B. melitensis 18, 22, 23 and the B. abortus 3, 10, 15, etc.). Irrespective of the initial H-ion concentration, 16 strains of the 21 B. melitensis studied produced a reduction equal to a P_H of 0.6-0.8, two strains of 0.9, one of 1.0 and two of 0.5. Of 20 B. abortus cultures tested, the reduction was: 18 a P_H of 0.7-0.8, and two of 0.9. This important and characteristic reaction emphazised by Miss Evans is therefore confirmed by our tests.

Indol is not produced in Difco-peptone solutions by any of our strains. Only B. melitensis strains 10 and 24 and B. abortus 80, 8, 32, 33 and 38 gave reactions in nitrate broth, which could be interpreted as indicating the presence of nitrites. Neither Horrocks nor Eyre for B. melitensis nor Fabyean for B. abortus were able to demonstrate a true reduction of nitrates to nitrites.

Neutral red and lead acetate agar give a slight growth with absence of a reduction of the dye or the chemical.

Following the suggestion of Miss Evans, our cultures were also tested for the production of ammonia in asparagin and urea containing mediums. All our strains of B. melitensis and B. abortus decomposed urea. B. melitensis strains 7, 9, 10 and 24 and B. abortus 80, 10, 34 and 40 produced a marked amount of ammonia, about equal to one mgm. in 20 c c of medium. On the other hand, the decomposition of asparagin was irregular and in comparison with the one in urea rather slight for most of the B. melitensis strains. In the only complete series in which all the actively growing B. abortus strains were tested simultaneously either no reaction or indefinite changes were recorded with Nessler's reagent. The following B. melitensis strains decomposed asparagin and gave a distinct ammonia reaction: B. melitensis 7, 9, 11, 23, 24, 25 and 26. Even vigorously growing strains of the genus "Brucella" may therefore fail to register ammonia production in asparagin solutions.

The viability of the cultures of B. abortus and B. melitensis in sealed tubes protected from desiccation and kept at a uniform temperature (18-22 C.) Eyre,[6] Mohler, and Traum [28] is well known. We were successful in obtaining viable cultures from agar slants of all the

[28] Twenty-Eighth Annual Report of the Bureau of Animal Industry, Department of Agriculture, 1911, p. 154.

strains which had remained unopened at room temperature for 6 and 10 months, respectively, after inoculation.

CONCLUSIONS

A comparative study of 21 cultures of so-called "Micrococcus" melitensis obtained from various sections of the world and of 32 cultures of B. abortus (Bang) isolated in this country and England justifies the following conclusions:

The causative organism of undulant fever of man and of malta fever of goats cannot be distinguished morphologically or biochemically from the organism responsible for infectious abortion in domesticated animals.

So-called "Micrococcus" melitensis appears in young cultures as a short rod and should therefore be designated as Bacterium melitensis.

The pigment production of the majority of actively growing B. melitensis strains on glycerol peptic digest agar and on alkaline potato cylinders after 5 days' incubation is more intense than with the strains of B. abortus.

Both B. melitensis and B. abortus cultures produce after 20 days' incubation in glucose and lactose broth an alkaline reaction and a characteristic reduction of the H-ion concentration equal to about 0.6 to 1.0 PH.

PRINCIPLES IN SEROLOGIC GROUPING OF B. ABORTUS AND B. MELITENSIS. CORRELATION BETWEEN ABSORPTION AND AGGLUTINATION TESTS

STUDIES ON THE GENUS BRUCELLA NOV. GEN. II

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A series of absorption tests, carried out with B. abortus and B. melitensis antiserums, resulted in a definite grouping of B. abortus and B. melitensis strains. A similar grouping ensued from a series of agglutination tests, in which eleven antiserums from rabbits, a monkey and a guinea-pig were used. The correlation, under diverse circumstances between the two sets of groupings, permits the inference that certain immutable laws govern serologic reactions, and although these laws remain elusive, we have been impressed by certain important principles involved in grouping.

Some preliminary tests with B. abortus and B. melitensis antiserums disclosed marked variations in the agglutinability of a number of authentic melitensis strains. In several melitensis antiserums, for example, the range of variability was so great that certain strains appeared to have little in common. Nevertheless, a relationship was apparent in that the strains of high titer in one antiserum perhaps group themselves as low titer strains in another antiserum. Thus while the variability was not identical, it was at least constant for the strains concerned. This suggested that definite principles might govern these variations. In the hope of elucidating these principles and of thus establishing the relationship among B. melitensis strains and their relationship to B. abortus strains, absorption tests were undertaken with abortus and melitensis antiserums. These experiments resulted in a four-fold grouping of the strains concerned.

PRELIMINARY DISCUSSION ON ANTIBODIES

The creation of antibodies is explained on the hypothesis that the bacterium consists of one or more components, each producing its specific agglutinins during the process of immunization. Biologically

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allied species may possess one or more components in common, hence the phenomenon of group or coagglutination. Castellani demonstrated that by saturating the antiserum with an emulsion of the coagglutinating organism, all these coagglutinins could be removed, leaving the specific agglutinins of the homologous strains intact. It was formerly believed that an antiserum could be exhausted of the specific agglutinins only by saturation with the homologous strain, but both Kruse and the co-workers, Andrewes and Inman, in their study of the serological races of the Flexner dysentery group, found that this was not the case. As the latter workers pointed out, "Of two strains A and B, A may be able completely to exhaust serum B, but B may be unable to do the same for serum A." Our own experience corroborates this point, and we have been able further to specify the condition under which occurs the phenomenon of a nonhomologous strain exhausting an antiserum, namely, when the two strains belong to the same serologic group, but are not identical, as will be demonstrated later.

In comparison to the stress laid on the structure of the bacterium in evoking the formation of antibodies, too little emphasis has been placed on the complexity of the serum itself. It is as if we mixed an acid in an alkaline solution and were to explain the process of neutralization by the composition of the acid regardless of the composition of the alkali. We find, for example, that the same bacterium may evoke the formation of quantities of antibodies in the serum of one species of animal, while it evokes but a feeble amount in a serum of another species;[1] yet we evade all responsibility of attempting to unravel the mystery from the point of view of the serum. We appease our curiosity with some selfevident platitude, such as the individuality of the animal, and thus veneer our ignorance with a gloss of logic. Why this individuality? Until serologic workers direct their investigations to unraveling the complexity of the serum, we cannot hope to fathom the marvelous mechanism of serologic reactions, nor can we offer any adequate explanation why a fourfold division of bacterial species results repeatedly from serologic classifications. Meningococcus (Gordon), pneumococcus (Cole and associates), tetanus (Tullock), Flexner dysentery (Andrewes and Inman),[2] typhoid (Weiss

[1] For example, one of our strains produced agglutinins to 1:20,000 in a monkey, and 1:2,000 in a rabbit.

[2] According to Andrewes and Inman, their "Y" or fifth race contains no specific antigenic component but presents a mixture of the component of the other four races. They say "we have failed fully to solve the antigenic structure of the 'Y' races. All that we have been able to do has been to obtain evidence of the presence in the race of most or all of the antigenic components which we have termed, V, W, X, and Z." (p. 36) Ref. 1.

and Hooker), streptococci (Dochez, Avery and Lancefield), influenza (Small-Dickson), and our own work on abortus-melitensis are included in this fourfold division. Is it not significant that the isolysins and iso-agglutinins of the human race also fall into four groups?

PRELIMINARY DISCUSSION ON ABSORPTIONS

In all our absorption tests we have proceeded on the principle that incomplete absorption of the nonspecific agglutinins, while it may yield interesting data, cannot establish definite laws. Unless the antiserum is absorbed to extinction of its nonspecific agglutinins, no subsequent reaction can be classed as distinctly specific. As a control, our absorbed serum was always tested with the absorbing strain along with our entire series of experimental organisms. If any agglutinins remained for the absorbing strain, the test was discarded. In practice we found it helpful to make a preliminary test with the absorbing strain, and if all of its agglutinins had not been removed the serum was reabsorbed. When the limit of extinction is reached, no further saturation with a nonspecific strain will effect a reduction in the titer of the serum for the specific agglutinins. On the other hand, as we have observed, an incomplete absorption, on becoming complete, may further reduce the specific titer 50%. It seems of vital importance, therefore, if we are to class residual agglutinins as specific that the nonspecific agglutinins should be absorbed to extinction.

Logically, the term specific is relative to the absorbing strain—for different bacteria may absorb different amounts and kinds, and in each case we term the residual agglutinins "specific." It may be possible that the definitely specific agglutinins of a bacterium can be measured only by successive saturations of its antiserum with different bacteria, each capable of removing quantitatively and qualitatively its own coagglutinins. But even then, there would be a minimum of residual agglutinins beyond which no bacterium except the homologous strain, or one of the same group, could exhaust the antiserum. We have not performed such tests and we use the term specific in its accepted sense of residual agglutinins after absorption with a nonhomologous strain.

TECHNIC

Antigens.—The strains were grown on peptic digest agar, the growth washed off with a few c c of formalinized salt solution and stored in the ice chest as stock emulsions. To 100 c c of formalinized salt solution the necessary amount of emulsion was added to match a standard in capacity containing about one

and one-half billion organisms. The same amount was added in each case whenever it became necessary to replenish the antigens. Although we use light suspensions throughout the experiments, we have since made use of opaque suspensions which have yielded excellent results. All agglutination readings were recorded after 18 hours in the incubator.

Method of Absorption.—The antiserum was diluted 1 : 10 with salt solution. An equal quantity of an emulsion of the absorbing antigen was added, making a dilution of 1 : 20. The tube was left 2 hours in the incubator and overnight in the ice chest, after which it was centrifuged one hour. The clear serum was decanted and a preliminary test made with the absorbing strain, to determine whether all of its agglutinins had been removed. If not, the serum was reabsorbed, using packed cells of the antigen in order not to alter the dilution of 1 : 20 The following day the clear serum was tested for agglutinins with 14 of our experimental strains, including the control. Thus we obtained evidence of relationship, not only between the absorbing and the homologous strains, but also for a number of other strains. Grouping at once became apparent.

Antiserums.—Although seven antiserums were employed in the course of our absorption investigations which covered over 400 tests, we submit the data from two of these, these two having been absorbed systematically by the greatest number of strains. Table 1 also contains the data of absorption tests from monkey antiserum. The magnitude of the task prevented us from pushing the absorption tests to completion in all the antiserums, but about 100 selected tests convinced us that the results paralleled each other. The antiserums of the classification submitted were made by immunizing rabbits, with both dead and living cultures, one with B. abortus 80, and the other with B. melitensis 7, these being our classical stock strains of B. abortus and B. melitensis.

RESULTS AND RECORDS OF ABSORPTION TESTS

It is quite evident that by absorbing an antiserum with one strain and then testing it with a number of other strains we may obtain one of three results. The coagglutinins may have been removed for all the remaining strains, thus giving a negative reaction throughout; they may have been removed for none of the remaining strains, thus giving a positive reaction throughout; or they may have been removed for some and not for others, thus giving a negative reaction for the former and a positive reaction for the latter. In the first and second cases we obtain no information as to grouping, all strains having followed an identical course. In the third case, however, a division into two groups is apparent. Let us assume, for example, that a certain serum absorbed by a certain strain was then positive for A, B and C, and negative for X, Y and Z, thus giving us two groups. Now another serum absorbed by the same strain, or if we choose the same serum absorbed by another strain, may leave A, B and Z negative, while X, Y and C are positive. We now have four groups. A and B have acted identically

throughout, and X and Y have clung together; C has fallen into a group by itself, and Z has done likewise. A third absorption with another serum or another strain may leave A and Y positive, and B, C, X and Z negative. We would now have as many groups as strains, for no two would have followed an identical course throughout.

Apparently this differentiation into groups is based on affinity for the same agglutinins. So long as two strains continue to follow parallel courses under various conditions, they are exhibiting like properties and may be assumed to possess similar components; for when the coagglutinins are absorbed from an antiserum for one of the strains, they are absorbed for the other; when the coagglutinins remain for the one, they likewise remain for the other. If this uniform behavior continues throughout a series of different types of tests, it is reasonably evident that the strains have a preponderance of something in common. Since they constantly react uniformly they naturally group themselves in the same categories.

At a special stage in our work, we became conscious of this group affiliation. Certain strains were exhibiting identical reactions (qualitatively) regardless of the antiserum or the absorbing strain used. There was, it is true, a quantitative difference—the titer when positive was higher or lower—but qualitatively they reacted in a uniform manner—their coagglutinins were either absorbed or were not absorbed under the same conditions. By checking the results, we found that the fourteen experimental strains fell into four groups with the greatest number in group 2.

These groups were as follows:

Group 1: Melitensis, 20; abortus, 80.

Group 2: Melitensis, 18, 19, 21, 2, 6, 8, 11, 655, 10.

Group 3: Melitensis, 7.

Group 4: Paramelitensis, 22; paramelitensis, 23.

Other strains tested irregularly distributed themselves in the various groups, the abortus strains invariably falling in group 1. Table 1 represents a portion of the data from which these groups were compiled. The results are expressed qualitatively (not quantitatively). The experimental strains are in the extreme left column and their reactions are to be read in a horizontal line. The strain and antiserum used in the absorption test are indicated at the top of the column and at the bottom, the groups to which they belong.

In endeavoring to analyze the principles involved in this grouping our data (as may be seen from the table) indicated that the strains

of one group could not exhaust the antiserum of another group; for example, no strain of groups 2, 3 or 4 could exhaust the antiserum of group 1. The antiserum of group 1 could be exhausted only by its homologous strain or by some other strain of group 1. This suggests that there is a specific component in each group which differentiates it from all other groups. On the other hand, it is apparently a common property for members within a group to exhaust the antiserum one

TABLE 1

Qualitative Records of Absorption Tests Representing the Four Groups of B. Abortus and B. Melitensis

									Rabbit
Agglutinated with	Anti-melitensis 7 Absorbed with Melitensis 7	Anti-abortus 80 Absorbed with Melitensis 7	Anti-melitensis 7 Absorbed with Melitensis 11	Anti-abortus 80 Absorbed with Melitensis 11	Anti-melitensis 7 Absorbed with Melitensis 18	Anti-abortus 80 Absorbed with Melitensis 18	Anti-melitensis 7 Absorbed with Melitensis 19	Anti-abortus 80 Absorbed with Melitensis 19	Anti-melitensis 7 Absorbed with Melitensis 21
Melitensis 2	0	+	0	0	0	0	+	0	0
Melitensis 6	0	+	0	+	0	+	0	+	0
Melitensis 7	0	0	+	0	+	0	+	0	+
Melitensis 8	0	+	0	+	0	+	0	+	0
Melitensis 10	0	+	0	0	0	Not tested	0	0	0
Melitensis 11	0	+	0	0	0	+	0	0	0
Melitensis 18	0	+	0	0	0	0	0	0	0
Melitensis 19	0	+	0	0	0	+	0	0	0
Melitensis 20	0	+	0	+	0	+	0	+	0
Melitensis 21	0	+	0	+	0	+	0	0	0
Melitensis 22	0	0	+	0	+	0	+	0	±
Melitensis 23	0	0	+	0	+	0	+	0	±
Melitensis 655	0	+	0	0	0	+	0	0	0
Abortus 80	0	+	0	+	0	+	0	+	0
	Group 3 absorbed with group 3	Group 1 absorbed with group 3	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2

+ signifies agglutination after absorption.
0 signifies no agglutination after absorption.
± signifies indistinct reaction.

for another without a reciprocal exhaustion taking place. Indeed in our limited investigations along this line, it was the prevailing case. We are not prepared to state that it is an obligatory relationship for one strain to be able to exhaust the antiserum of another strain in the same group, but we suspect that such may be the case. This implies a close relationship among the allied strains of the same group.

The second point revealed was that if a strain exhausted an antiserum of its coagglutinins for some strain in another group, it exhausted the coagglutinins for all strains in that group under the same absorption conditions; that is, the action was uniform (qualita-

tively) on the entire group. If the reaction were positive, the same principle applied. To illustrate the foregoing points, let us glance at the above grouping and suppose that strain 18 removed from antiserum 7 the coagglutinins for strain 20 (group 1), but not for strain 22 (group 4). Then strain 18 also removes the coagglutinins from antiserum 7 for strain 80 and for all other members of this group, but does not remove them for strain 23 or for any other members of this

TABLE 1—*Continued*

Qualitative Records of Absorption Tests Representing the Four Groups of B. Abortus and B. Melitensis

Antiserums						Monkey Antiserum					
Anti-abortus 80 Absorbed with Melitensis 21	Anti-melitensis 7 Absorbed with Paramelitensis 22	Anti-abortus 80 Absorbed with Paramelitensis 22	Anti-melitensis 7 Absorbed with Paramelitensis 23	Anti-abortus 80 Absorbed with Paramelitensis 23	Anti-melitensis 7 Absorbed with Melitensis 20	Anti-abortus 80 Absorbed with Melitensis 20	Anti-melitensis 7 Absorbed with Abortus 80	Anti-abortus 80 Absorbed with Abortus 80	Anti-melitensis 655 Absorbed with melitensis 7	Anti-melitensis 655 Absorbed with Abortus 80	Anti-melitensis 655 Absorbed with Melitensis 655
0	+	+	+	+	±	0	±	0	+	Not tested	0
+	Not tested	+	Not tested	+	±	0	±	0	+	+	0
0	+	?	+	0	+	0	+	0	0	0	0
+	+	+	+	+	±	0	±	0	+	+	0
+	Not tested	+	Not tested	+	±	0	±	0	+	Not tested	0
+	+	+	+	+	±	0	±	0	+	+	0
−	+	Not tested	Not tested	+	±	0	±	0	+	+	0
0	+	+	+	+	±	0	±	0	+	+	0
+	+	+	+	+	0	0	0	0	+	0	0
0	+	+	+	+	±	0	±	0	+	+	0
0	0	0	0	0	+	0	+	0	0	0	0
0	0	0	0	0	+	0	+	0	0	0	0
+	+	+	+	+	±	0	±	0	+	+	0
+	+	+	+	+	0	0	0	0	+	0	0
Group 1 absorbed with group 2	Group 3 absorbed with group 4	Group 1 absorbed with group 4	Group 3 absorbed with group 4	Group 1 absorbed with group 4	Group 3 absorbed with group 1	Group 1 absorbed with group 1	Group 3 absorbed with group 1	Group 1 absorbed with group 1	Group 2 absorbed with group 3	Group 2 absorbed with group 1	Group 2 absorbed with group 2

group. We see, then, that a strain acts in a uniform manner on every member in another group, under the same absorption conditions.

We observed further (but we are not prepared to state this as a universal fact) that all strains in one group were likely to act in the same manner (qualitatively) on all members in another group when absorbed from the same antiserum. For example, continuing the above illustration, our data revealed that strains 19, 21 and 11 (same group as 18) also removed the coagglutinins from antiserum 7 for strains 20 and 80, but did not remove them for strains 22 and 23. The same principle asserted itself when five strains of group 2 were absorbed from group 1 antiserum. Here the reaction was positive for strains 20 and 80, and negative for strains 22 and 23 in all 5 cases.

We may state this tentatively as follows: All strains in one group tend to act in the same manner (qualitatively) on all strains in another group, when absorbed from the same antiserum. If this is true, there is a uniform action of group on group, which is more than our principle advocates, namely, the uniform action of each strain on the entire group.

Immediately a third principle manifested itself as an amendment to the preceding. Occasionally a group did not act in unison, but analysis revealed the definite condition under which this deviation occurred; namely, an absorbing strain might act in an irregular manner on members of its own group, thus bringing out their individual differences. For example, strain 18 when absorbed from antiserum 80 removed the coagglutinins for itself and for strain 2, but did not remove them for the remaining strains of the same group; that is, we have a mixture of positive and negative reactions for members of the same group when subjected to the same absorption conditions. It may be that the difference among members within a group is purely a quantitative one, that each possesses a preponderance of the specific agglutinins but varying amounts of the foreign coagglutinins, and this difference becomes manifest only when one of the group acts as the absorbing agent in removing the coagglutinins. It must be borne in mind that while a group absorbs irregularly for its own members the whole group is acted on uniformly by members of another group.

So far as we carried our experiments, we found no deviation from these three principles. We attempted to check our results by the following test: One of the workers planted from his own private stock 6 of the experimental strains and gave them to the other worker under fictitious lettering. In all 6 cases the strains were assigned to their proper groups and in 4 cases the exact organism was located. The latter point, however, is beyond the scope of our work. We cannot scientifically separate one strain from another in the same group, and the ability to do so is merely temporary and due to that intangible evidence which constant handling of a strain brings to a worker.

The three principles enunciated above may be briefly summarized:

1. An antiserum cannot be exhausted by strains of another group. It is always exhausted by its homologous strain and may be exhausted by other members of the same group.

2. A strain acts in a uniform manner (qualitatively) on all strains in another group under the same absorption conditions. This uniform action constitutes the basis for group affiliation.

3. Strains within the same group do not necessarily act in a uniform manner on one another under the same absorption conditions. This constitutes the basis for individual differentiation.

The degree of demarkation between any two groups is far from uniform. There is a wide difference between groups 1 and 3 and between groups 1 and 4, but the relationship between groups 1 and 2 is exceedingly close, and generally they follow identical courses only to be separated when some specific element comes into play. Frequently the strains of these groups interagglutinate to their full titers. Group 2 appears to be a transition between groups 1 and 3. It bridges their differences; it is related to both, but it is sharply separated from group 4. Group 3 may be taken as the type of the classic melitensis strain, from a serologic standpoint. Its agglutinins are in a large measure specific, and it is clearly defined from other types. Group 4 embraces strains of low agglutinability and of low antibody producing powers. They are fairly agglutinable in some antiserums if living suspensions are used, but all our work was carried out with formalinized suspensions. They appear to have few agglutinins in common with groups 1 and 2; they have a more pronounced absorbing effect in group 3 than their agglutinating titer might intimate. But agglutinating titer is not a criterion for absorbing titer. In comparing the percentage agglutinating with the percentage absorbing titer for the different groups, it was found that the percentage absorbing power of a group was frequently in excess of its percentage agglutinating power. The reverse was rare. On the whole, group 3 appears to be the pivot from which the strains radiate in one direction toward the abortus group, and in the other direction toward the highly specific paramelitensis group.

It has been stated that whenever B. abortus strains were tested they fell in group 1. We carried but one abortus strain, number 80, throughout all the tests. Into the same group fell melitensis 20. Under all absorption conditions these two strains followed identical courses except that there was a quantitative difference in titer. Melitensis 20 could exhaust the antiserum of B. abortus 80 and toward the end of our work when we prepared an antiserum for melitensis 20, we found that it could be depleted by B. abortus 80, thus giving a reciprocal exhaustion. We then reduced the absorbed dilution to 1 : 10, and 4 other B. abortus strains were now absorbed from melitensis 20 anti-

serum. All 4 exhausted it. One of these strains had been carried through about half the absorption test. We now found that melitensis 20 could exhaust its antiserum. Thus we had two B. abortus strains which could exhaust the antiserum of melitensis 20 and whose antiserum melitensis 20 could exhaust. Nevertheless, the 3 strains are not identical, so far as their histories go. Their titers, though approximate, are not identical in all antiserums. Their coagglutinins are not equally removed quantitatively by other strains. They grow differently both as regards speed and abundance. We are, therefore, forced to conclude that reciprocal exhaustion in dilutions as low as 1:10 is no criterion for identity of strains. This sounds illogical, but it does not exclude the possibility that specificity may be demonstrable in dilutions of 1:2 or 1:5. All that we conclude from these reciprocal absorption tests is the close relationship between B. abortus and one type of B. melitensis strains. What then is specificity? The fact that a nonhomologous strain can exhaust an antiserum casts a doubt on individual specificity as the exclusive possession of a single bacterium. It would appear that no bacterium is an isolated entity, all of its agglutinins provoked by immunization, are shared by some members in its group and out of its group. Each group may possess a separate primary attribute, but not each bacterium, and the sum total of these primary attributes constitutes the race; for example, the race of typhosus, of dysenteriae, of pneumococcus. The individuality of a bacterium would then appear to consist in its proportional share of the agglutinins of its race — the primary group agglutinins predominating — rather than in the possession of a specificity exclusively its own.

Andrewes and Inman,[3] in their masterly article on the Flexner dysentery types, used a quantitative method in their absorption tests. They determined the number of bacteria in their absorbing emulsions and diluted these for two absorbing doses, one containing approximately 1,000 million organism, and the other from 20,000 to 30,000 million organisms. They advocate a quantitative method. We have not been convinced of the advantage in determining the number of organisms in the absorbing dose—except its interest from the experimental point of view. It would be vexatious in routine work even if emulsions were kept in stock, and moreover its result might be fallacious. If our principle is correct that the absorbing strain must

[3] Medical Research Committee Special Report No. 42, 1919.

remove all the coagglutinins it is capable of absorbing — that is, to extinction of itself—there could be no fixed doses for any one strain, because its absorbing capacity varies with the potency of the serum and its relationship thereto.

When dense doses are required there seems to be less hindrance to the progress of the reaction, if absorbed fractionally. This, however, is not obligatory.

GENERIC CLASSIFICATION

The American Committee on the Classification of Bacterial Types [4] decided that "B. abortus may for the present be left in this genus (Bacterium) in spite of its peculiar oxygen relations." The genus Bacterium of the Bacteriaceae family constitutes the colon-typhoid-dysentery group. It would seem that this genus is already encumbered with sufficiently diversified types without the addition of B. abortus.

If in reality a classification is a scheme destined to convey some adequate idea of mutual relationships, should not its genera be so apportioned that each genus may be narrowed to a type, embracing individuals with fairly limited common characteristics and common differentiations from other types; thus, one genus should not include organisms with such widely varied specificity as B. coli, B. typhosus, B. dysenteriae and B. abortus, although all these would still be united in a common family. If, as is the case in the above genus, the "species" is left as the sole vehicle for differentiation (for the term subgenus is a useless encumbrance), a classification becomes an empty nomenclature, a mere vocabulary with which the sophisticated student may terrify the uninitiated scholar.

We advocate, therefore, that B. abortus be removed from the genus Bacterium, which includes the colon, typhoid and dysentery organisms, and we suggest that the abortus melitensis group be given separate rank as the genus "Brucella" (from Bruce who isolated the original melitensis organism, later identified by Nègre and Raynaud, as Micrococcus paramelitensis.[5]) The 4 groups as above formulated would then each embrace a number of allied species, and if it became expedient to establish subgroups, they would probably range themselves as varieties of some species.

[4] Jour. Bacteriol., 1917, 2, p. 546.

[5] Compt. rend. Soc. de biol., 1912, 72, p. 791 and 1052.

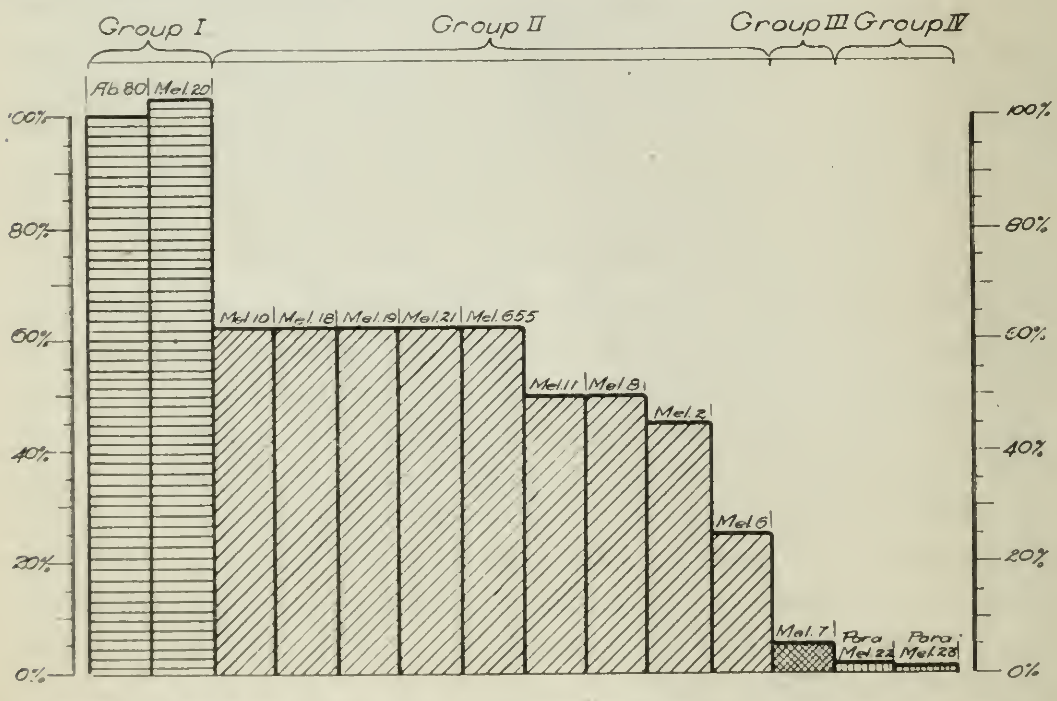
RESULTS AND RECORDS OF AGGLUTINATION TESTS

The experimental strains were agglutinated in 1 monkey, 1 guinea-pig and 9 rabbit antiserums. All 4 groups were represented by these 11 antiserums. We also tested the strains in the serums of 2 cows and 3 hogs suffering from natural abortion disease. Separate references will be made to these tests.

In addition to the experimental strains used in the absorption tests, we carried 15 other B. melitensis strains through all the tests and 35 B. abortus strains were agglutinated in 6 of the antiserums.

B. abortus and B. melitensis unlike B. typhosus, for example, are not strong antibody producers. Our B. abortus antiserums ranged from 1:2,000 to 1:4,000 and our B. melitensis antiserums approximated 1:2,000 except that

Chart 1.—Agglutinogenesis of group 1 antiserum. Columns of like marking represent different strains of the same group.



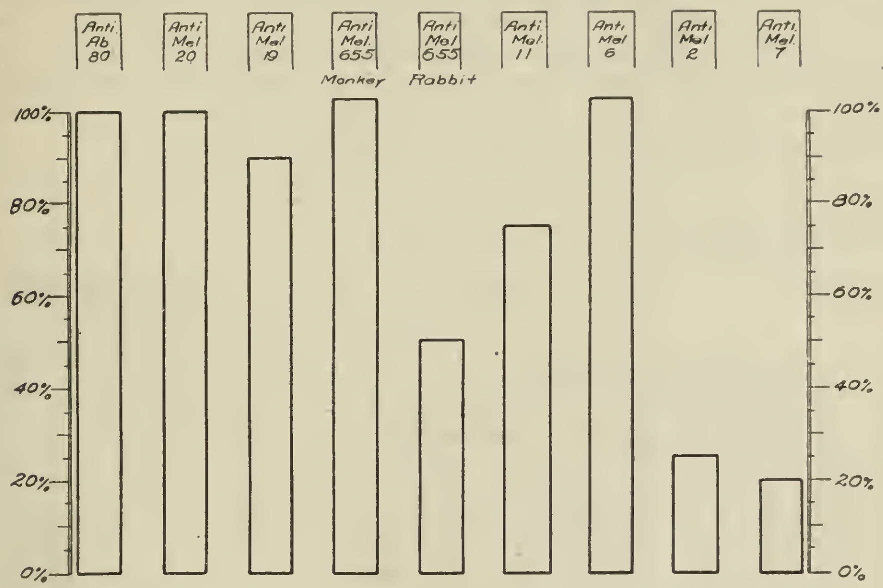
Abortus 80 antiserum (group 1). Percentage agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

of the monkey which was active in a dilution of 1:20,000 and the so-called paramelitensis of group 4 which did not yield an antiserum beyond 1:200 in either a rabbit or a guinea-pig.

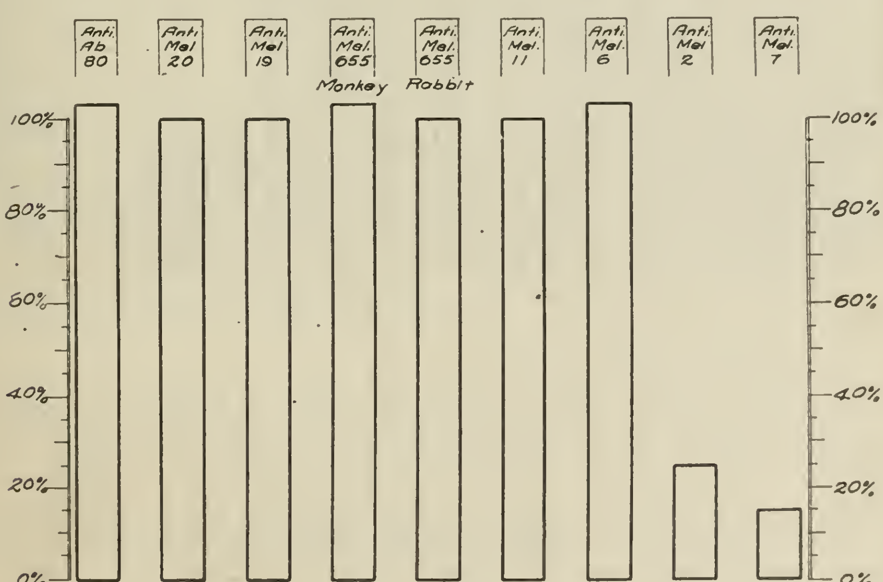
In expressing our results graphically, we have adopted the column and percentage method used by Andrewes and Inman.[3] As these workers pointed out, actual figures are not comparable owing to the different titers of various antiserums, whereas results are readily comparable if expressed as percentages of the titers for the homologous strains. The titer for the homologous organism is taken as 100% and the proportional titers for others are expressed as percentages of this. Thus, if a strain reacts to 1,000 in an antiserum which flocculates the homologous organism to 2,000, the former's titer is expressed as 50%. Occasionally a strain reacts beyond the titer of the homologous organism, in which case the percentage is expressed above the 100 mark.

In presenting the results which follow we shall discuss first, the action of the antiserums of each group on the various strains, and then the agglutination of the strains of each group in the various antiserums.

Chart 2.—Agglutination of group 1 strains in 9 antiserums. Compare columns above with columns below for action of same antiserum on two strains of group 1.



Percentage agglutination of abortus 80 bacterium (group 1) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

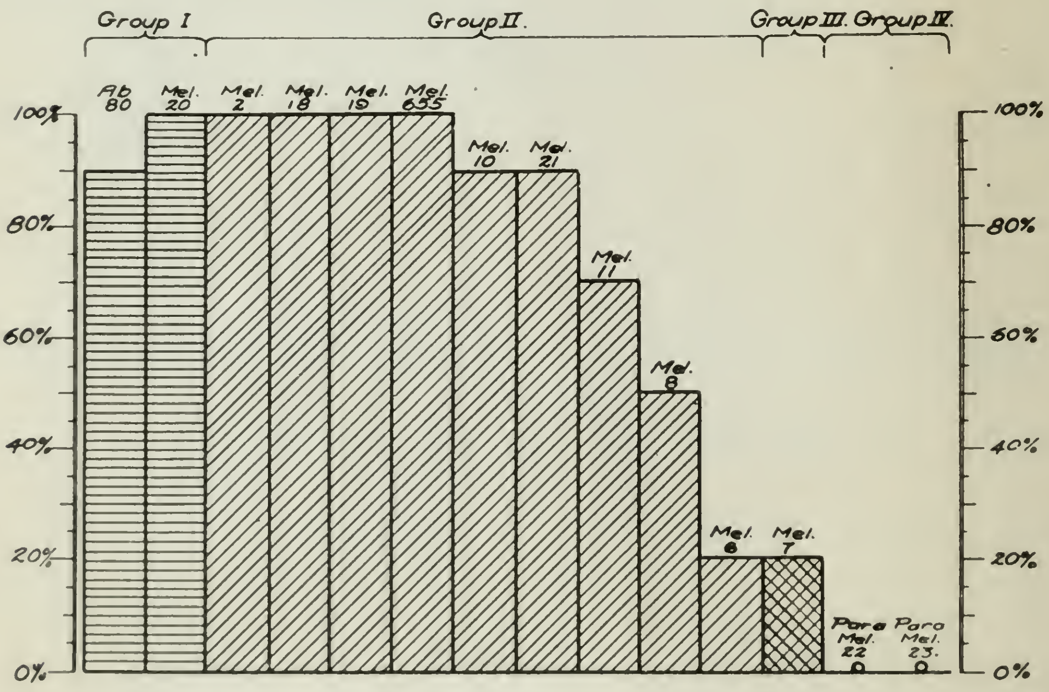


Percentage agglutination of Melitensis 20 bacterium (group 1) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

Group 1 Antiserums.—A potent antiserum of this group was characterized by definite gradations in the titer limits for the strains of the four groups. Group 1 strains agglutinated 100%. Group 2 averaged 60%, but in some of

the less potent antiserums, such as those not exceeding 1:2,000, these strains agglutinated to the full titers, thus making no distinction in agglutinability for groups 1 and 2, a situation which will repeat itself later on. Group 3 strains did not react beyond 5%, and group 4 strains 1% or less. In those antiserums in which group 2 strains reacted to the full titer, there was no proportional change in the titers for groups 3 and 4. Chart 1 represents a potent antiserum of group 1 showing the percentage agglutination for the experimental strains. In this antiserum the agglutination of group 2 averages 60%. Chart 9 represents the more common antiserum of group 1 in which the group 2 strains are flocculated to the titer limits. Strains 2 and 6 are somewhat irregular in most antiserums, but the absorption tests assign them to group 2.

Chart 3.—Agglutinogenesis of group 2 antiserum (rabbit). Columns of like markings represent different strains of the same group.



Melitensis 19 antiserum (group 2). Percentage of agglutination for 14 strains. .The titer for the homologous strain is 100 per cent.

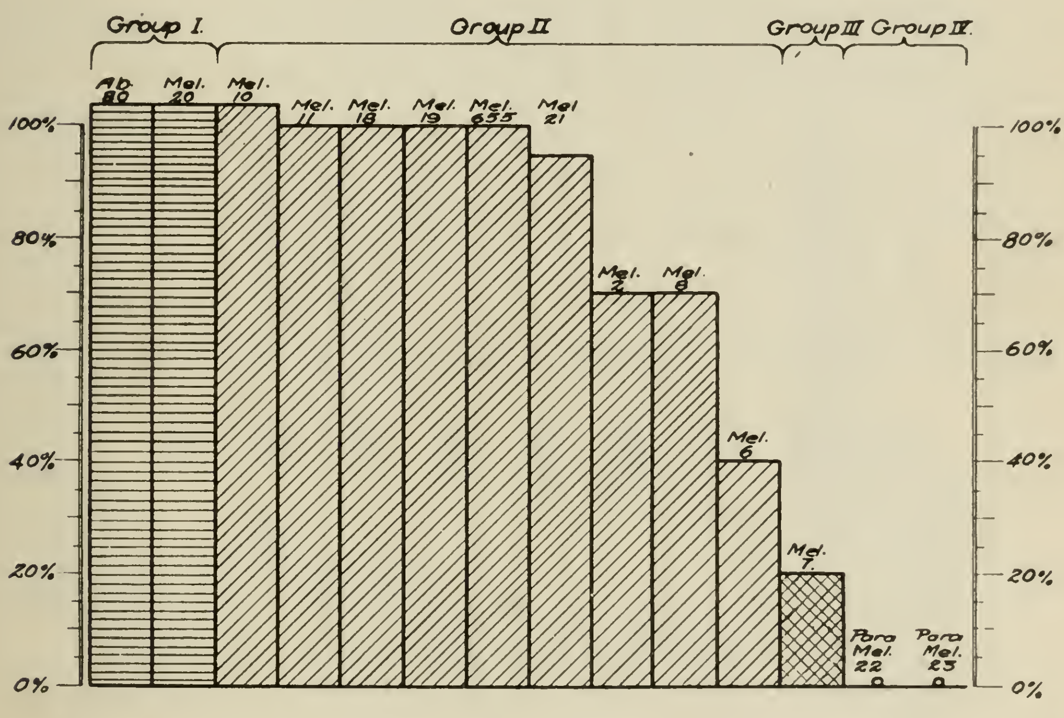
The 35 abortus strains showed a uniform range of 100% or thereabouts in all antiserums of group 1 and apparently represent a uniform group. Chart 10 shows these B. abortus strains in a group 1 antiserum.

Group 1 Strains.—Whatever may be the inhibitory forces which prevent a group 1 antiserum from flocculating the strains of groups 3 and 4 to any marked degree, the same forces are in evidence when the strains of group 1 are agglutinated in the antiserums of groups 3 and 4. About 40 B. abortus strains and B. melitensis 20 of group 1 could not react beyond 20% in group 3 antiserum. As group 4 (so-called paramelitensis) did not yield an antiserum in excess of 1:200, we limited our tests in this antiserum. Its agglutinins for group 1 fell below its titer. Group 2 antiserums generally agglutinated group 1 strains to close to the titer limits. It will be seen that B. melitensis 2 anti-

serum was not very potent for group 1 strains, showing a similar irregulariy to that of group 1 antiserum for melitensis 2 strain. Chart 2 shows the agglutination of 2 unselected strains of group 1 in 9 antiserums.

Group 2 Antiserums.—These antiserums generally show no fundamental difference in the titers for strains of groups 1 and 2. They do not necessarily flocculate all strains to the titer limits, but the difference in degree of agglutinability is not sufficiently pronounced to establish a basis for separating the groups. Miss Evans'[6] y f strain—our B. melitensis 11—was of this group and from the reaction of its antiserum she concluded that "the agglutination reactions in Bacterium melitensis antiserum can distinguish Bacterium abortus from

Chart 4.—Agglutinogenesis of group 2 antiserum (monkey). Columns of like markings represent different strains of the same group.



Melitensis 655 antiserum (monkey), group 2. Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

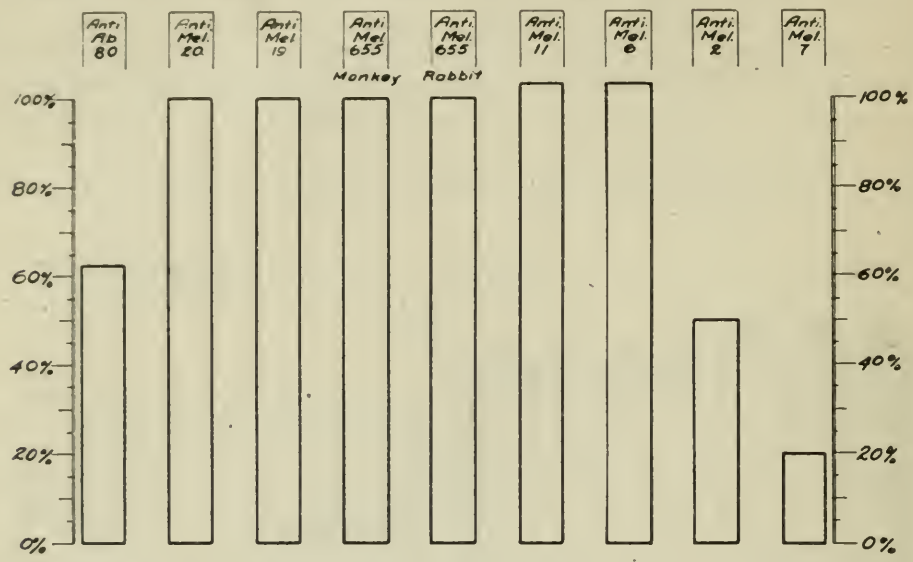
Bacterium melitensis only when the agglutinating strength of the serum for both species is known." Other workers (Kennedy[7]) who have found a close relationship in the reactions for both species, were probably working with group 1 strains.

It can be stated that whenever there is reciprocal agglutination approximating the titer limits in abortus and melitensis antiserums, the homologous strains must belong either to group 1 or to group 2. These groups cannot be separated from each other by absorption with a group 3 or a group 4 strain. They follow similar courses in such cases. They can be differentiated by reciprocal absorptions with their own group strains and antiserums. The distinction is gen-

[6] Jour. Infect. Dis., 1918, 22, p. 580.
[7] Jour. Roy. Army Med. Corps, 1914, 22, p. 9.

erally very delicate. All strains in this group are not equally agglutinable. Strains 2, 6 and 8 may fall considerably below the titer limits. Indeed the repeated irregularities of strains 2, 6 and possibly 8 (for which we had no

Chart 5.—Agglutination of group 2 strains in 9 antiserums. Compare columns above with columns below for action of same antiserums on two strains of group 2.



Percentage of agglutination of melitensis 18 bacterium (group 2) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

Percentage of agglutination of melitensis 655 bacterium (group 2) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

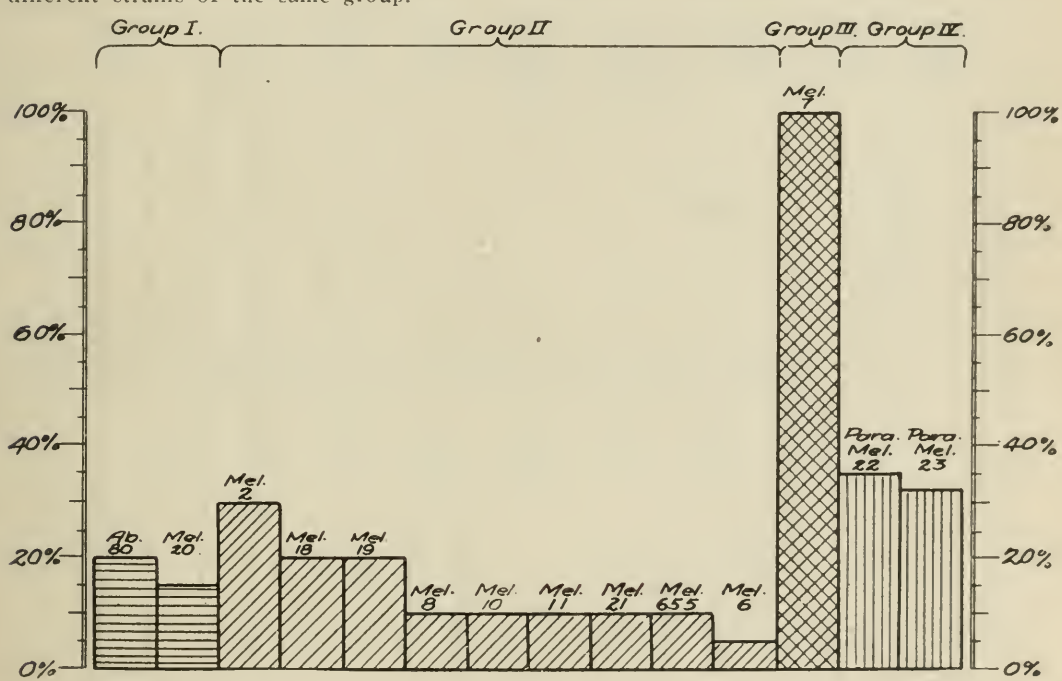
antiserum) suggest that their antigenic structure, if investigated, might justify placing them in a subdivision of group 2. Separately treated they would not distort the uniformity of the group nor distract the mind by constant reference to their irregularities. The titer of group 2 antiserum for group 3 strain

averages 20% except in antiserum 6 where it reacts to the titer limit. There are no demonstrable agglutinins in group 2 antiserums for group 4 strains except in antiserums 2 and 6 where there is a slight reaction.

Charts 3 and 4 show the titers of two antiserums of this group for the experimental strains. Chart 3 is a rabbit antiserum with a titer of 1:2,000 and chart 4 a monkey antiserum with a titer of 1:20,000. It will be seen that the gradations are fairly uniform in the two antiserums in spite of the striking differences in their titers and the fact that the immunization was made with 2 different strains of group 2.

Group 2 Strains.—As stated above, the strains of group 2 are agglutinated to about 60% or may be flocculated to 100% in group 1 antiserum. In group 3 antiserum they agglutinate from 10% to 20% of the full titer. Thus again we

Chart 6.—Agglutinogenesis of group 3 antiserum. Columns of like marking represent different strains of the same group.



Melitensis 7 antiserum (group 3). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

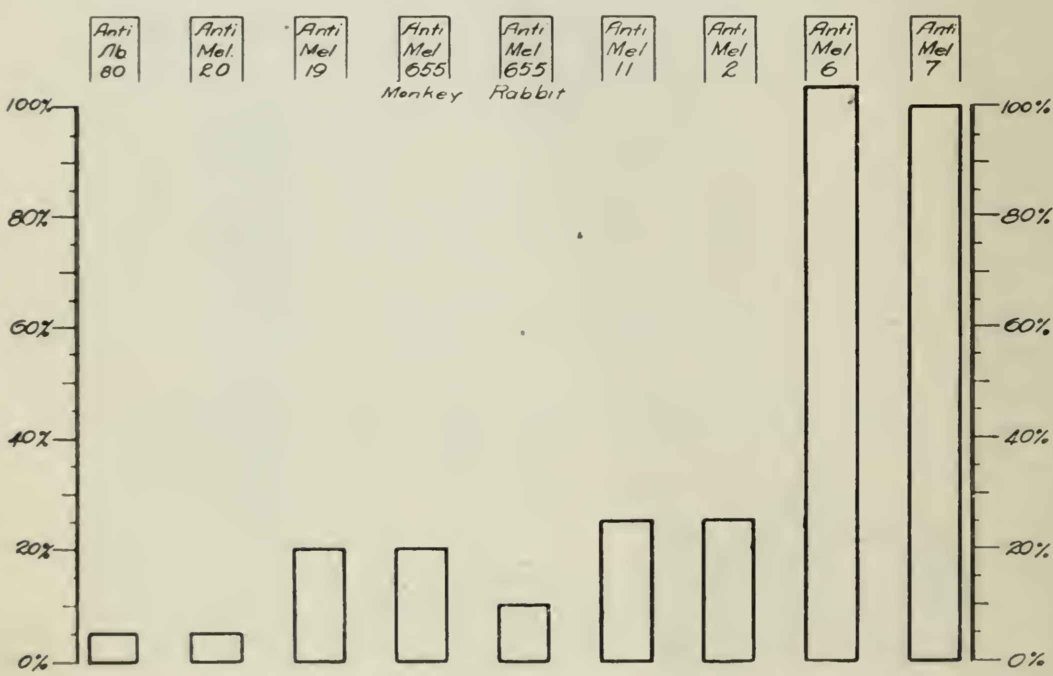
see the same inhibitory forces at work, confining the reaction of a group 2 antiserum on a group 3 strain and conversely the reaction of a group 3 antiserum on a group 2 strain to about 1/5 of their respective titers. In the weak antiserum of group 4 all strains of group 2 reacted to 1:200 which was the titer limit. Chart 5 shows the percentage agglutination of 2 strains of group 2 in 9 antiserums.

Group 3 Antiserum.—The foregoing summaries have necessarily overlapped the reactions for groups 3 and 4. The agglutinins for group 3, which measured 100% for itself, are low for strains of groups 1 and 2, averaging about 1/5 of the titer limit. Melitensis 2 runs somewhat higher and melitensis 6 slightly lower than the other strains. Groups 1 and 2 cannot be separated from each other by agglutination in group 3 antiserum though they can be differentiated instantly from group 3 itself. On the other hand, group 3 is the only antiserum in which there is a fair agglutination for strains of group 4. These

readily react to at least 30% of the titer. As living suspensions they may agglutinate to 100% in this antiserum. Chart 6 shows the percentage agglutination of group 3 antiserum for the experimental strains, and chart 9 for the series of B. abortus strains which reacted from 10% to 20%.

Group 3 Strain.—This strain (our 7) probably the M. pseudomelitensis of Sergent, Gillot and Lemaire,[8] reacts to 5% in group 1 and to about 1/5 of the titer in group 2 antiserums, and hence it is readily separated from them both. Melitensis 6 antiserum is a striking exception. It agglutinates group 3 to its titer limit. This is especially irregular because the antiserum of group 3 shows its minimum reaction on strain 6. Group 3 strain reacted to the full titer of 1:200 in the group 4 antiserum. Chart 7 shows the percentage agglutination of a group 3 strain in 9 antiserums.

Chart 7.—Agglutination of group 3 bacterium in 9 antiserums.



Percentage of agglutination of melitensis 7 bacterium (group 3) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

Group 4 Antiserum and Strains.—This low titer antiserum did not show any differential reaction for the various groups except that group 1 ran slightly below the titer. The strains of this group are inagglutinable in most antiserums. They show a slight reaction in group 1 antiserums and in melitensis 2 and melitensis 6 antiserums of group 2 in addition to their reaction in group 3 antiserum.

It will be seen from the subject matter presented above that we obtain striking gradations in agglutinability whether we consider the reaction of the antiserum on strains of the different groups or the reaction of strains in the antiserums of different groups. Moreover, these gradations coincide with the groups established by the absorption tests. We may briefly summarize these gradations:

[8] Ann. de l'Instit. Pasteur, 1908, 22, p. 209.

Group 1 antiserum does not generally differentiate between the strains of groups 1 and 2, though in an occasional potent antiserum it may do so. It agglutinates group 3 weakly and is agglutinated weakly by group 3. It shows a minimum reaction for group 4.

Chart 8.—Agglutination of group 4 strains in 9 antiserums. Compare columns above with columns below for action of same antiserums on two strains of group 4.

Percentage of agglutination of paramelitensis 22 bacterium (group 4) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

Percentage of agglutination of paramelitensis 23 bacterium (group 4) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

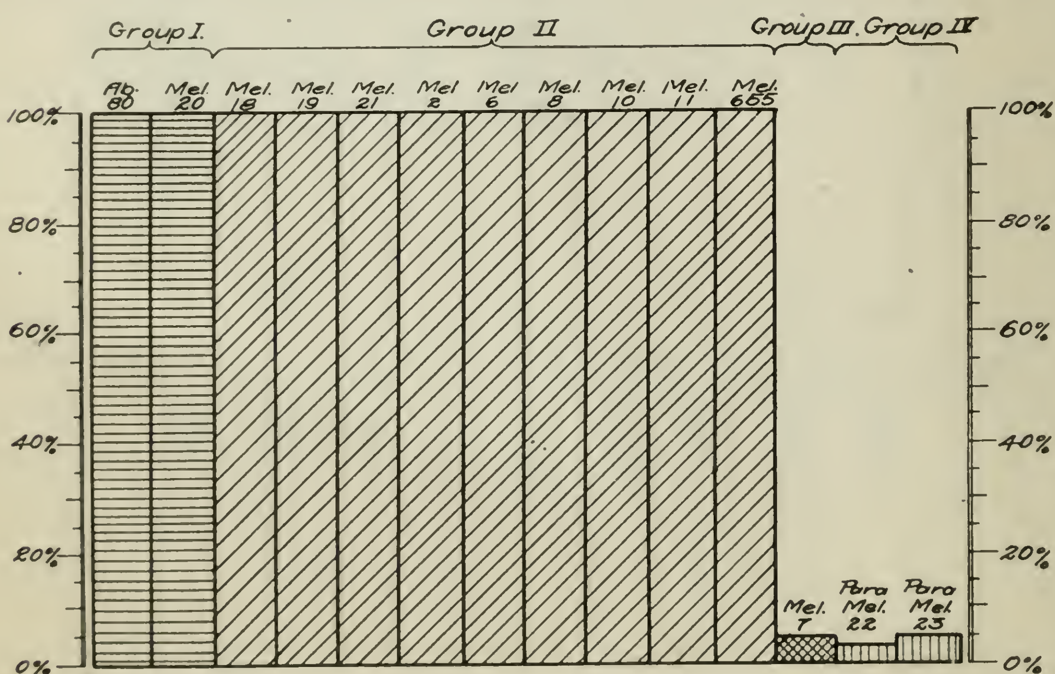
Group 2 antiserum shows no vitally specific differentiation between the strains of groups 1 and 2. It acts weakly on group 3 (except in antiserum 6) and is weakly acted on by group 3. It has no agglutinins for group 4 except in antiserums 2 and 6.

Group 3 sharply differentiates itself from groups 1 and 2, and is equally sharply differentiated by them (except in antiserum 6). It agglutinates group 4 to at least 30% of its titer.

Group 4 is differentiated by its prevailing inagglutinability in most antiserums and its inability to produce a potent antiserum in either rabbits or guinea-pigs.

The studies of Sergent and his co-workers,[8] who reported finding "para" and "pseudo" melitensis strains, probably foreshadowed our grouping. Nègre and Raynaud[5] identified one of Bruce's original organisms and gave it the name of paramelitensis. They also reported a race intermediate in agglutination between the "para" and the true melitensis.

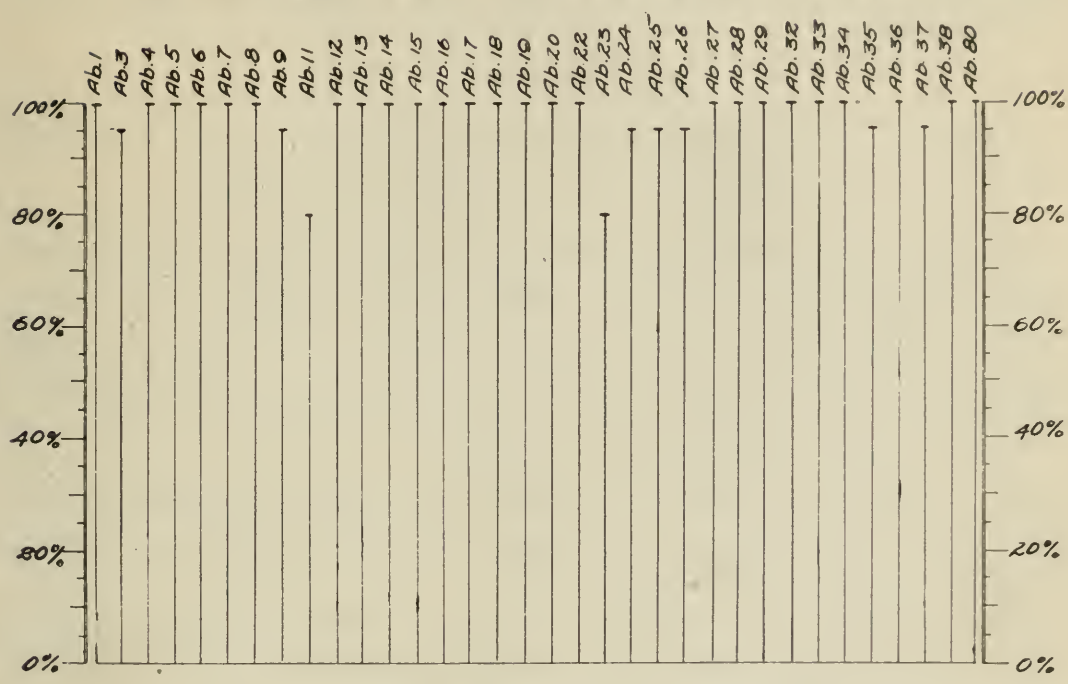
Chart 9.—Agglutinogenesis of group 1 antiserum. Columns of like marking represent different strains of the same group.



B. melitensis 20 antiserum (group 1). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

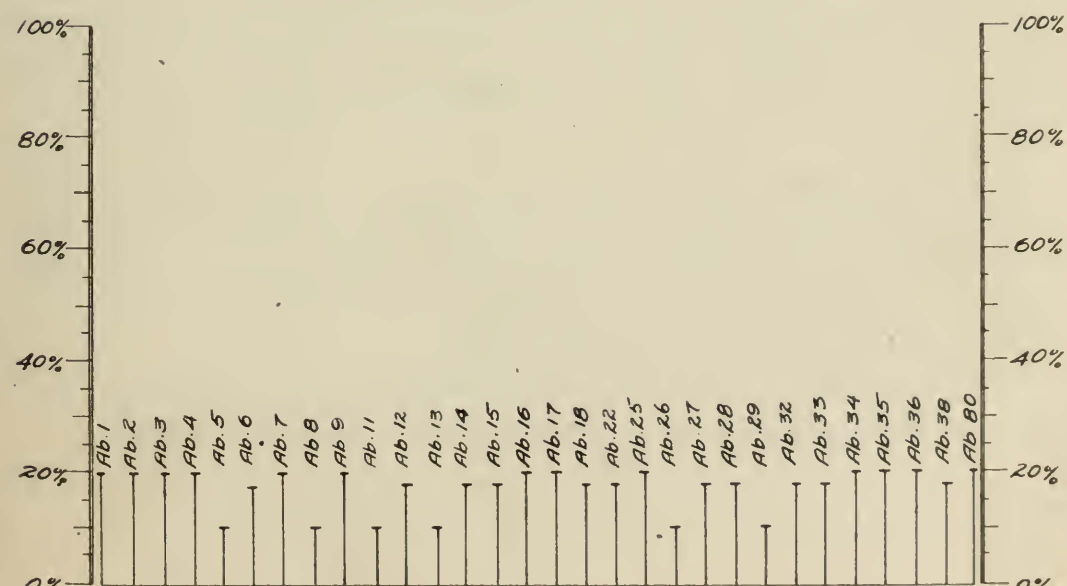
Through the courtesy of Dr. J. Traum, University of California, we obtained antiserums from 2 cows and 3 hogs suffering from natural abortion disease. All these serums, except that of one cow, reacted to 1:200 with B. abortus. The excepted cow showed no reaction to B. abortus nor to B. melitensis strains. There was, however, in unheated serum a faint sedimentation with both group 4, the so-called paramelitensis strains. The other cow, on the contrary, yielded an antiserum which flocculated all strains of groups 1 and 2 to the titer limit, but showed no reaction for strains of groups 3 and 4. The 3 hog antiserums flocculated B. abortus and showed a weak reaction for one or more B. melitensis strains, that is, 2 of the hogs gave a reaction for but one (not the same) B. melitensis strain, and the third for 6 melitensis strains. Group 3 was not agglutinated by any of the 5 antiserums. It would seem that the animals, except one cow, were infected by a group 1 strain, the abortus group. From

Chart 10.—Agglutination of B. abortus strains in group 1 antiserum.



B. abortus 14 antiserum (group 1). Percentage of agglutination for B. abortus strains. The titer for the homologous strain is 100 per cent.

Chart 11.—Agglutination of B. abortus strains in group 3 antiserum.



B. melitensis 7 antiserum (group 3). Percentage of agglutination for B. abortus strain. The titer for the homologous strain is 100 per cent..

the above data we see that in immune serums naturally or artificially produced we obtain a reaction for both B. abortus and B. melitensis strains, which corroborates fully the observations of Evans[6] and Kennedy[7] with bovine serum and milk whey.

SUMMARY

Unless an antiserum is absorbed to extinction of the absorbing strain, the residual agglutinins cannot be classed as specific.

A series of absorption tests with formalinized suspensions in B. abortus and B. melitensis antiserums led to a fourfold grouping of 14 B. abortus and B. melitensis strains. Groups 1 and 4 were represented by 2 and group 3 by 1 strain, the majority fell in group 2. All B. abortus strains belonged serologically to group 1. Groups 1 and 2 are closely related. They are sharply defined from groups 3 and 4.

The grouping revealed these principles:

1. An antiserum cannot be exhausted by strains of another group. It is always exhausted by its homologous strain, and may be exhausted by other members of the same group.

2. A strain acts in a uniform manner (qualitatively) on all strains in another group under the same absorption conditions. This uniform action constitutes the basis for group affiliation.

3. Strains within the same group do not necessarily act in a uniform manner on one another when absorbed from the same antiserum. This constitutes the basis for individual differentiation.

In conforming to the main classification adopted by the Society of American Bacteriologists, we suggest that B. abortus and B. melitensis group be given generic rank in the Bacteriaceae family as the genus "Brucella."

A series of agglutination tests in B. abortus and B. melitensis antiserums disclosed gradation in titer limits for the different strains and the gradations were constant for the same strains in the various antiserums. It was found that the sets so formed correlated with the groups resulting from the absorption tests.

The serums of cows and hogs suffering from natural abortion disease may also react to both B. abortus and B. melitensis organisms.

FURTHER STUDIES ON "BLACKHEAD" IN TURKEYS, WITH SPECIAL REFERENCE TO TRANSMISSION BY INOCULATION

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Although the parasite causing the disease in turkeys popularly known as "blackhead" was discovered many years ago, the mode of its transmission has not been satisfactorily demonstrated. Several distinct problems are involved in this question of transmission: (1) the source of the parasite; (2) the route by which it enters the body of the turkey, and (3) its pathogenic properties or virulence.

Whether the parasite of blackhead, Histomonas meleagridis, occurs only in association with the disease, and is thus to be regarded as uniformly pathogenic, or is widely prevalent in the cecal contents of normal turkeys, and only invades the tissues under conditions which lower the host's resistance, has, up to the present, been regarded as a more or less open question. The possibility that this organism, while pathogenic for the turkey, may occur commonly in the intestinal tract of other species, such as the common fowl in which it may rarely produce lesions, should also be considered. The determination of any of these various points concerned in the transmission of blackhead may prove of practical importance in the rearing of turkeys.

Source.—Considerable information is already available as to the sources of the infection. That the disease may be acquired by the exposure of normal turkeys to those which have shown evidence of infection, has been experimentally demonstrated by Theobald Smith,[1] and Tyzzer.[2] Smith [3] concludes that turkeys which have passed through an attack are more dangerous as sources of infection than are those showing symptoms of active disease. Blackhead occurs chiefly in young turkeys during the summer months, and acute cases are often lacking throughout the greater portion of the year. It is obvious, therefore, that other sources of infection exist. We are not warranted, however, in assuming from Smith's results that the acute

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[1] Jour. Med. Res., 1915, 33, p. 243.
[2] Jour. Med. Res., 1919, 40, p. 1.
[3] Jour. Exper. Med., 1917, 25, p. 405; 1920, 31, p. 633.

case is negligible as a source of infection, for mere exposure to infected turkeys — probably on account of the numerous uncontrolled factors in the procedure — has furnished notably variable results. An instance of the transmission of the disease from turkeys with acute cases to a normal turkey will be presented below. It is not known how long the parasite may persist in turkeys after recovery from blackhead, but it is to be suspected that such birds may serve as "carriers" and transmit the disease to the young of the succeeding season.

The common fowl, although it rarely shows lesions characteristic of blackhead, should also be regarded as a probable source of the infection. Smith has recorded an outbreak of blackhead in a brood of young turkeys not exposed to other turkeys, but confined near a hennery. The appearance of the disease in two lots of young turkeys following exposure to a flock of common fowls in which the disease was known to have been present earlier in the season, has been noted by Tyzzer — although the conditions of the experiment were not as carefully controlled as desired. Blackhead has also been observed by this author in a brood of young turkeys on a small farm where there were hens but no other turkeys. The view is frequently expressed that young turkeys should be raised on new, uncontaminated soil, but there is at present no reliable data available to show how long the virus remains alive outside the body.

Mode of Entrance of Parasite.—The lesions of blackhead as it occurs naturally are usually confined to the ceca, liver and contiguous surfaces to which the lesions may extend. Since the parasite evidently first invades the cecal wall and later reaches the liver by way of the portal system, it appears probable that it enters through the alimentary tract by the ingestion of contaminated food or water.

There is apparently a widespread belief that damp surroundings or wettings are fatal to young turkeys, and that wet seasons are especially unfavorable. The following extract from "Josselyn's Voyages—An Account of Two Voyages to New England, by John Josselyn, Gent., London — Printed for Giles Widdows at the Green Dragon in St. Paul's Churchyard 1674"— indicates the antiquity of this belief and also suggests the possible occurrence of blackhead in early colonial days. On page 99 appears this passage:

"The turkie, which is in New-England a very large Bird, they breed twice or thrice in a year, if you would preserve the young chickens alive, you must give them no water, for if they come to have their fill of water they will drop away strangely, and you will never be able to rear any of them: they are

excellent meat, especially a Turkie-Capon beyond that, for which eight shillings was given, their eggs are very wholesome and restore decayed nature exceedingly. But the French say they breed the Leprosie: the Indesses make coats of Turkie-feathers woven for their children.

The Partridge is larger than ours, white flesht, but very dry. They are indeed a sort of Partridges called Grouses, etc."

An outbreak of blackhead in turkeys is often attributed to a storm or wet weather immediately preceding the appearance of symptoms, but this is disproved by experiments showing that an incubation period of at least ten or twelve days is necessary before symptoms appear. That cold, damp surroundings and an occasional wetting will not of themselves produce blackhead has been amply demonstrated by the authors' experience in rearing turkeys without loss from disease under such conditions. Normal turkeys acquire it only when the virus of the disease is available; as for example, by exposure to diseased or convalescing birds or to "carriers." Thus, although the parasite of blackhead is undoubtedly widely distributed, it is probably not ubiquitous and is evidently not present in normal young turkeys. Whether dampness and wet weather favor infection by lowering the resistance of the host or by favoring the transmission of the parasite has not yet been demonstrated. If the parasite proves to be readily destroyed by drying, the greater frequency of infection in wet weather is explained by the favorable conditions for its tranmission, rather than by the lowering of the resistance of the turkeys.

The feeding of blackhead lesions to normal turkeys has frequently resulted negatively. The question of the presence of living virus in such material, the possibility either of its passage through the intestinal tract without entering the ceca, or its destruction by normal digestion, or that some special mechanism is required for it to penetrate the mucosa of the ceca, all furnish problems for speculation, as nothing is definitely known concerning these points. The exposure of normal to infected turkeys is also productive of variable results, and on account of its uncertainty, cannot be relied on as an experimental procedure.

Pathogenicity.—Until the disease can be produced at will, it is difficult to draw any definite conclusions as to the presence of infective virus, its pathogenicity, or to determine the susceptibility of various species suspected of being sources of infection. When it is possible to produce the disease constantly with a given virus, the properties of the latter may be determined, such as its resistance to various external conditions, as well as to various drugs therapeutically administered to

the infected host. Such experiments should also throw light on the question of the incubation period, the possibility of unrecognized attacks, latent infection, "carriers," etc., and also on the pathology of blackhead.

Experiments which were carried out during the summer and autumn of 1919, have served primarily to demonstrate the uniform susceptibility of turkeys, and the relative nonsusceptibility of a number of other species of birds and of laboratory mammals to the inoculated disease. In addition to this: (a) the infectiousness of the inoculated disease has been demonstrated; (b) an attempt has been made to transmit the disease through the agency of "blow flies"; (c) confirmatory evidence relating to the common fowl as a source of the disease has been obtained; and (d) attempts have been made by the administration both of tartar emetic and of quinin to destroy the parasite in the tissues of the inoculated turkey.

The virus employed in the inoculation experiments was derived primarily from the liver lesions of freshly killed young turkeys in the acute stage of the disease, but was subsequently taken from the lesions of inoculated birds. Turkeys, chickens, pigeons, rabbits, guinea-pigs and mice were inoculated. The method of inoculation usually employed was to implant, by means of an inoculation trocar, several milligrams of material obtained from active lesions, either beneath the skin or in the muscle. The turkeys were hatched in the laboratory and reared in an enclosure outside; young chickens, and three quarter grown White Leghorns were obtained from eggs hatched in an incubator at the laboratory, and large Rhode Island Red chickens were purchased; pigeons were obtained in the open market and with two exceptions were of the common variety; and the mammals employed were all laboratory stock animals. The turkeys were reared under the following conditions.

Eggs, obtained on May 31, from a flock in which blackhead had occurred during the previous season, were incubated under common hens until they commenced to hatch, when they were transferred to an incubator. Of the 24 eggs incubated, 3 were sterile, 1 was crushed near the time of hatching, and 20 young turkeys were hatched on June 27. One with a large unabsorbed yolk sac died several days later. Within 48 hours after hatching, these turkeys were placed outdoors in a small brooder house opening out into a small wire enclosure. When about a fortnight old they were allowed the run of the yard in which turkeys had been reared and exposed to blackhead the previous summer.

On August 9, forty-three days after hatching, 2 of the largest of the lot were found dead, evidently suffocated as the result of the entire flock crowding under a sloping board platform during an unusually cold night. Neither of these 2 birds showed any lesions of the internal organs. Three days later the flock suffered another loss, one turkey having been taken and another fatally injured by a cat. In order to avoid further depredations, the turkeys were subsequently confined daily in the brooder house and attached wire enclosure between 5 p. m. and 8 a. m. until they were 2 months old, when a larger cat-proof enclosure was constructed for them to stay in during the night. For 8 hours during the day they were allowed the run of the entire yard. The space available for forage amounted to about 15 sq. yds. for each turkey. With confinement each night in the brooder house, together with continual wet weather, the feathers became badly soiled and the feeding habits became much less cleanly than those of turkeys having a free range. Their appearance improved as soon as the larger enclosure was provided and a satisfactory rate of growth was maintained in these somewhat limited quarters (see chart 1). The diet consisted of mixed grain, grit, sour milk, and an abundance of green forage, chiefly dandelion. Under these conditions 15 young turkeys were reared for the experiments reported below.

On account of the number and similarity of the experiments, and of the results obtained, the detailed protocols of only a few illustrative cases will be presented. The results of the inoculations will be discussed with reference to the different species employed, while the more important data obtained in the various experiments will be furnished in part by a chart showing the growth curves of the inoculated turkeys and in part by tables.

INOCULATED BLACKHEAD OR HISTOMONIASIS

Turkey.—The subcutaneous inoculation of young turkeys with bits of fresh liver lesion from an acute case of blackhead, has produced a characteristic and invariably fatal disease. Seven turkeys inoculated in this manner on one occasion, and others inoculated successively later on, have either succumbed to the disease or were in a dying condition when killed. The following case will illustrate the course of the inoculated form of the disease:

TURKEY 19-1.—Aug. 14, 1919: Weight, 550 gm.; inoculated subcutaneously in the left breast with a bit of liver lesion from Turkey C. P. S. 19.48 (obtained from a small flock several of which had already succumbed to blackhead).

Aug. 22: Weight, 690 gm.; an indurated mass, elevated, with well defined border, but not more than 1 cm. in its greatest diameter, was apparent at the site of inoculation. Several yellowish opaque areas in its substance were apparent through the skin.

Aug. 23: Rectal temperature at 12:30 p. m. 108.2 F.

Aug. 25: Inactive and rather weak. The breast showed a rather diffuse swelling, 2 x 3 cm. in diameter, with the muscle extensively involved. (Sulphur colored droppings noted in yard.)

Aug. 26: The lesion showed a more definite border and measured 3.5 cm. in length. The muscle appeared to be involved. The skin was intact and only slightly adherent to the surface of the lesion.

Aug. 29: Weight, 550 gm.; the lesion was roughly about one-half the size of a hen's egg and somewhate flattened; extreme weakness. Coughing had been noted among the inoculated turkeys for several days. (Since involvement of the lungs was not anticipated in the turkeys first inoculated, respiratory symptoms were not at first recorded for each individual turkey.)

Aug. 30: Found dead. Weight, 520 gm.; the subcutaneous mass measured 6 x 4 x 3 cm., and presented a central necrotic portion measuring 2 x 1.5 cm., of a firm dry texture and of a color ranging from yellowish to dull pink. Peripherally the mass consisted of grayish somewhat translucent tissue. The adherent breast muscle was reddened to a variable extent and was of soft consistence. The right lung contained a nodule (1 cm.) of firm, yellow tinged material surrounded by reddened edematous, lung tissue. There were also several ill-defined grayish lesions. The left lung showed more or less confluent, indefinite, grayish lesions, of soft consistence and without evidence of necrosis. The liver presented 6 small rounded lesions (3-4 mm.) with sharply defined borders, color pinkish, opalescent, mixed with yellow. Heart, kidneys and spleen appeared normal.

Histology.—Stained sections of the subcutaneous lesion showed an irregular, sharply defined, necrotic portion surrounded by a layer of inflammatory tissue, in places 1 cm. or more in thickness, for the most part distended with innumerable parasites. The necrotic portion was composed in part of dense appearing hyaline substance, and in part, of a reticulum of fibrinoid material, the interstices of which were filled with polymorphonuclear leukocytes, moderate numbers of other cells and parasites. The denser necrotic material was surrounded by a layer of giant cells. The peripherally situated inflammatory tissue showed dilatation of the blood vessels. There was also an infiltration with cells chiefly endothelial in type, with occasional polymorphonuclear leukocytes and small collections of lymphoid cells. The parasites were either free or within cells, the latter assuming the character of giant cells in the older portions of the lesions. At the periphery of the lesion the muscle fibers were widely separated with parasites and infiltrating cells. Many of the muscle fibers appeared swollen, stained faintly, and in some instances were disintegrating. The arrangement of the parasites indicated that they had replaced the muscle fibers.

In the sections of lung, the lesions showed the essential characteristics of the subcutaneous lesion. The most prominent feature was the infiltration of the tissue with large numbers of parasites. There was a small necrotic portion and at the periphery a well defined zone of cell infiltration. The interlobular portion of the lung was first invaded by the parasites leaving the infundibular portions relatively free. The air spaces of the affected areas showed large numbers of cells containing parasites and a small amount of serofibrinous exudate.

The liver lesions showed microscopically necrotic parenchyma intermingled with collections of organisms, and resembled those of spontaneous blackhead. Numerous giant cells were present.

The disease was transmitted by inoculation from turkey to turkey through 6 transfers, and was propagated in this manner from Aug. 14 to Nov. 7, a period of 85 days, without any diminution in its virulence. Its general character was maintained through the successive transfers.

Although it showed a remarkable regularity in its course, in some cases the respiratory symptoms were more marked than in others, and one showed symptoms suggesting involvement of the nervous system.

The primary lesion develops as the result of the multiplication of parasites at the site of inoculation. The implant is readily distinguished from the first as a small flattened nodule palpable through the skin, and usually shows no appreciable increase in size up to the sixth day. At this time or soon after, there is a well defined swelling of the tissues around the nodule which becomes indurated and subsequently increases rather rapidly in size. There is no tendency to ulceration and the skin has remained intact in every case, although these subcutaneous lesions have attained great size — in one instance measuring 8x4x1.5 cm. The nodule, which is never more than slightly adherent to the skin, is often firmly adherent to the breast muscle and may involve it to a variable extent as the lesion enlarges. It has an even rounded contour and is invariably flattened (see fig. 1). Its outer surface presents a centrally situated depressed area of opaque, dull yellowish or grayish color, and bulging, rounded border. The nodule is of firm consistence and on section shows a central, apparently necrotic, portion composed of dry caseous material varying in color from grayish to dull pink or yellowish (fig. 2). Around this, except for the external depressed area, is a layer of opalescent, slightly pinkish or reddish tissue which is thickest at the border of the nodule. Occasionally, there is a more or less, bright yellow, gelatinous exudate infiltrating the surrounding tissues.

In several of the inoculated turkeys the subcutaneous lesions have failed to attain great size and at the time of death appeared to consist of little more than a hard, flattened mass of necrotic tissue. It became evident in the course of the observations that the development of the subcutaneous lesion had no appreciable effect on the growth and health of the turkey, the progress of the disease depending on the involvement of vital organs.

Histologically, the primary lesion shows a central necrotic portion with irregular extensions into the surrounding tissue. In this are necrotic muscle fibers and obliterated blood vessels, but the tissues are, for the most part, replaced with a reticulum of fibrinoid material the interstices of which are filled with polymorphonuclear leukocytes and parasites, many of which are degenerated. The necrotic portion frequently shows concentric zones indicating its increase from time to

Fig. 1.—Subcutaneous lesion of turkey 19·7, which died 18 days after inoculation; × ½.

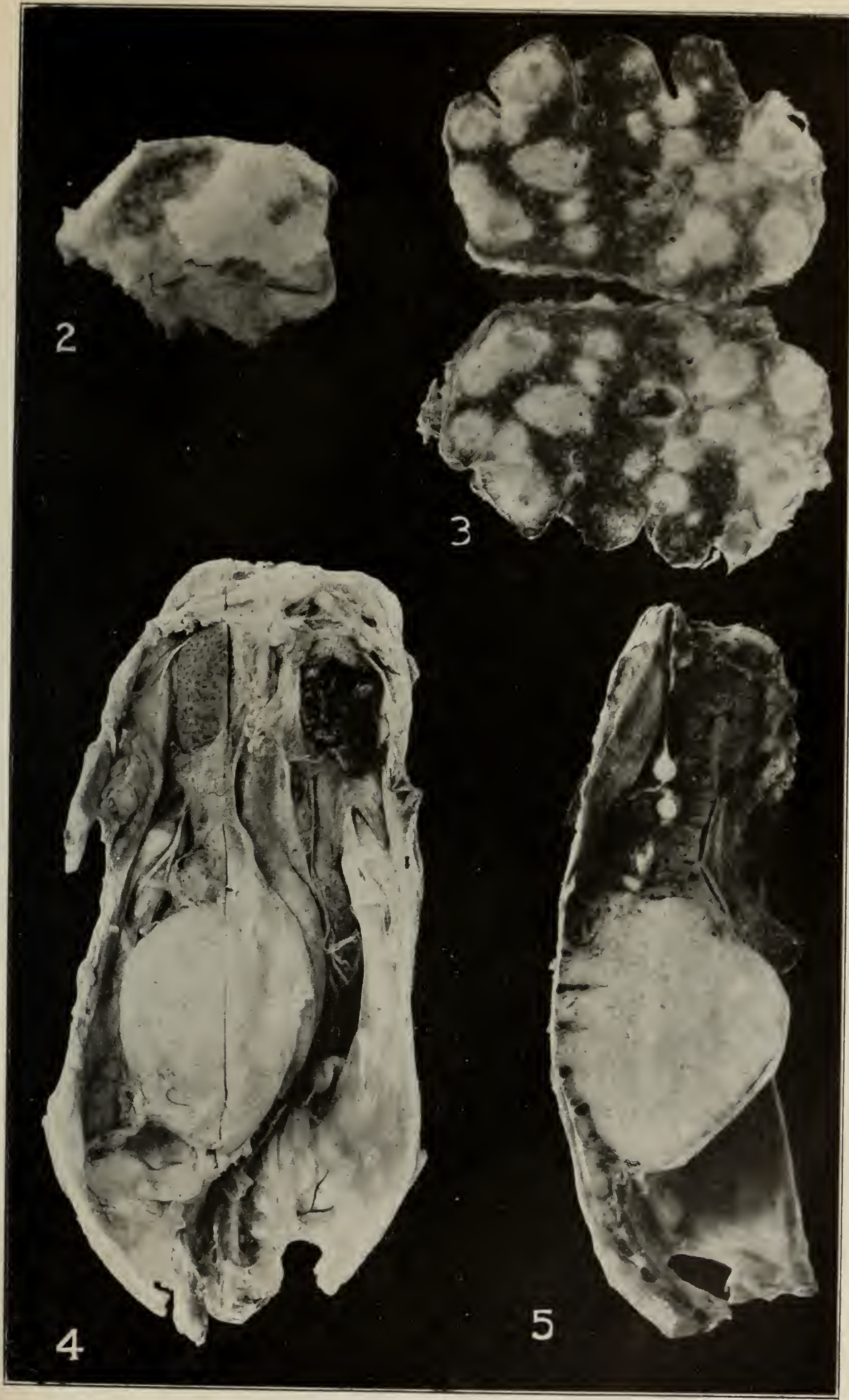

Fig. 2.—Cut surface seen on vertical section of a portion of the subcutaneous lesion of turkey 19-1. Central necrotic portion represented by light area above and to right, infiltrated peripheral zone to left of this and invaded breast muscle below; × 1½.

Fig. 3.—Surfaces of sectioned lung of turkey 19-10 killed on showing dyspnea 13 days after inoculation. The whitish areas represent focal lesions. The prominence of the lobular markings is due to a general perivascular and peri-infundibular infiltration; × 1.

Fig. 4.—Ventral aspect of dissection showing a large lesion of the right kidney of turkey 19-2, killed 25 days after inoculation. The surface of the uninvolved anterior extremity of the kidney is apparent above; × 1.

time with the progressive obliteration of blood vessels. The living tissue of the lesion is distended with parasites so that it virtually constitutes a reticulum. The blood vessels are dilated. The striated muscle fibers at the periphery of the lesion are swollen, stain less intensely, appear fragmented and are evidently disintegrating.

The reaction to the parasites consists chiefly of their phagocytosis by the endothelial cells which eventually form giant cells. The latter react not only to the parasites, but also to the necrotic tissue, around which they form a layer sequestrating it from the living tissue. In addition to the endothelial cell reaction, there is a rather marked infiltration of the necrotic tissue with polymorphonuclear leukocytes, but these cells are rare elsewhere except in the vicinity of blood vessels. Eosinophils and lymphoid cells occur in variable numbers at the border of the lesion. The reaction of the tissues evidently checks the multiplication of the parasite. Certain lesions studied consist to a large extent of massed giant cells in which nearly all the parasites are included. In some cases the protective reaction has gone so far as to reduce the lesion to a sequestrum of necrotic material surrounded by actively forming granulation tissue. It is a notable fact that considerable numbers of well preserved parasites persist in the spaces of the fibrinoid reticulum after they have disappeared from the living tissue of the lesion.

The pulmonary lesions vary in appearance in accordance with their size. The larger lesions, some of which measure 2 cm. or more in diameter, are composed of centrally situated, firm, dry, dull yellowish or pinkish material, and an external layer of soft, gelatinous, grayish tissue, with a more or less reddened periphery (see fig. 3). Smaller lesions are of soft consistence, of grayish color with reddening of the surrounding lung tissue and show little or no necrosis. The macroscopic characteristics of the larger lesions are thus quite similar to those of the subcutaneous lesion. The degree of lung involvement varied in different cases, but was extensive in all inoculated turkeys examined. The amount of lung tissue capable of functioning appears in some instances to have been reduced to a minimum. In extensively involved lungs, the lesions often become confluent, sometimes forming in the larger turkeys caseous masses 4.5 cm. in diameter. In a turkey weighing 2,020 gm., one of the lungs which was extensively involved weighed 45 gm. The process is found at times extending from the larger lesions to the thoracic wall with production of lines of opaque

induration in the intercostal muscles, extending parallel with the ribs. Attention was at first directed to the study of the subcutaneous lesions of the inoculated turkeys, and the lungs were not examined with special care in the first two turkeys to succumb. Each of the other 12 showed extensive lung involvement.

Microscopic examination of the lungs showed a process similar in many respects to that found in the subcutaneous lesion but modified by anatomic structure. The older portions of the lesions consisted of necrotic lung tissue and more or less fibrinoid reticulum enmeshed in which were numerous polymorphonuclear leukocytes and variable numbers of the parasite. Necrotic blood vessels were greatly distended with blood. As the parasites migrate from the early foci, they first infiltrate the peripheral zone of the lung lobule, and to a less extent the portions adjacent to the infundibular space so that a median zone of the lobule is for a time uninvolved. Usually most of the parasites that occur in the infundibular or bronchial spaces are included within cells, but in one case in which there was a rapidly developing dyspnea, the larger air spaces as well as the tissue showed great numbers of the blue staining, invasive forms. The cellular reaction in the involved lung is similar to that seen in the subcutaneous lesion, but there is, in addition, a variable amount of serous and fibrinous exudate into the air spaces. In fact, extensive pneumonia may frequently occur in portions not infiltrated by the parasite. The pneumonic lung may show edema and infiltration of the alveolar walls associated with atelectasis. The pulmonary lesions resemble the primary lesion in respect to the giant cell reaction to the parasite and necrotic tissue and to the peripheral cellular infiltration. Pneumonia may cause death in cases in which the parasite has been for the most part destroyed, so that it appears that the involvement of the lung is on the whole more fatal than that of the liver.

Lesions of the liver were found in 8 of the 14 turkeys inoculated, which is relatively infrequent as compared with their occurrence in the natural disease. In one of these turkeys only a single liver lesion was found, in none were there more than 6 present, and in the entire series a total of only 28 lesions was noted. These nodules were usually much smaller than the larger of the lung foci, but in two instances they were of equal size. The small size of the majority of the liver lesions indicates that they are for the most part secondary to the larger lung lesions; the occasional larger ones probably result from the simultaneous invasion of the liver and lung. It is quite evident

that the liver lesions are of the same general character in both the natural and the inoculated disease, so that further description of them in the latter is unnecessary. The distribution of the lesions in this infection is evidently determined by the factors governing the dissemination of the parasites, which will be discussed later.

Three of the inoculated turkeys developed lesions of the kidney. In one there was a large tumor-like nodule measuring 4x3.3x2.5 cm. in diameter (see figs. 4 and 5). This nodule had a smooth, rounded surface, and on incision appeared to be composed of soft, dull pinkish tissue, without markings. In its gross appearance the substance of this nodule simulated closely lymphoid tissue or that of a lymphoma. The kidney lesions in the other two cases were considerably smaller and appeared in the surface of the kidney as grayish, slightly elevated areas. From the diffuse character of the lesions and the abundance of organisms present, it is apparent that the kidney furnishes a favorable medium for the development of the parasite of blackhead.

The kidney lesions all show microscopically an active acute process. Although they may attain a much greater size than the lesions of the lungs and liver (see fig. 4), they differ from these and from the subcutaneous lesion in that there are only microscopic foci of necrosis, and fibrin is present in small amounts or absent. In one case the kidney appeared normal at postmortem examination, but showed a microscopic lesion in stained section. Early lesions show infiltration with great numbers of the pale, blue staining invasive forms of Histomonas, with a consequent crowding apart and destruction of tubules. Loss of parenchyma is associated with edema of the connective tissue. The cellular reaction is here as elsewhere, chiefly endothelial in type, and giant cells which are present in the early lesions, later occur in great numbers. Numerous endothelial cells are found in the process of mitotic division. Eosinophils occur in variable numbers chiefly around degenerating tubules. Leukocytes of other types are few in number. The parasites may occasionally be found within tubules, both free and within giant cells. They are distributed singly or in closely packed masses throughout the tissue, but do not occur in nests as in the cecal lesions.

The brain and spinal cord of one of the inoculated turkeys which developed a peculiar gait, with legs straightened and stilt-like, body carried horizontally, and wings held so that the back appeared flattened — were examined, but there was no macroscopic lesion to account for the peculiar symptoms.

Symptoms.—The period of incubation is found to be remarkably constant notwithstanding great discrepancy as to age and size of the various turkeys at the time of inoculation. Several of the 7 turkeys that were inoculated at the age of 48 days — when they weighed from 350 to 510 gm. — showed a slightly longer incubation period than the last one which was inoculated when 104 days old, and weighed 2,670 gm. Seven of the 14 turkeys inoculated showed the first symptoms 11 days later, two 12, two 14, two 15, and one 17 days later. It is evident that symptoms make their appearance only after vital organs have become extensively diseased. The first sign to be noted is a tendency to lag behind the rest of the flock, evidently a symptom of weakness. This is soon followed by more pronounced weakness, as shown by the slow careful gait with drooping wings, and the tendency to stand in one spot with the head under a wing. Other symptoms may appear at once or later, and without any regular sequence. Loss of appetite frequently appears almost simultaneously with the earliest sign of weakness. In some cases the appetite is capricious and varies from time to time — one turkey showed an abnormally voracious appetite. Loss of weight is practically concomitant with loss of appetite, and appears to occur invariably in the inoculated as well as in the natural disease. The effects of the inoculated disease on the growth of the turkeys employed, together with other data are illustrated in the appended chart.

During the summer of 1919, as well as in 1918, no loss of weight was recorded except in cases of blackhead. Sulphur colored droppings do not appear as regularly in the inoculated as in the natural form of the disease, but were noted in several of the inoculated turkeys. Since they have appeared in cases of inoculated blackhead, in none of which was there involvement of the cecum, it is obvious that they result from the derangement of the function of some other organ. It is probable that this change in the character of the excrement is in some way dependent on the involvement of the liver, but additional data are necessary to definitely establish this point.

Pulmonary symptoms appear rather late in the course of the disease produced by inoculation and amount at first to a slight cough which is increased by unusual exertion. A pronounced dyspnea develops in some cases late in the disease. The turkey then squats with beak and throat widely distended, making it possible to view the interior of the trachea through the distended larynx. Pronounced

dyspnea was not noted in many of the series, although the majority showed extensive involvement of the lung. Paleness of the skin about the head may become marked as the disease progresses, and in several was noted with the earliest sign of weakness. The earliest death resulting from the subcutaneous inoculation of blackhead virus thus

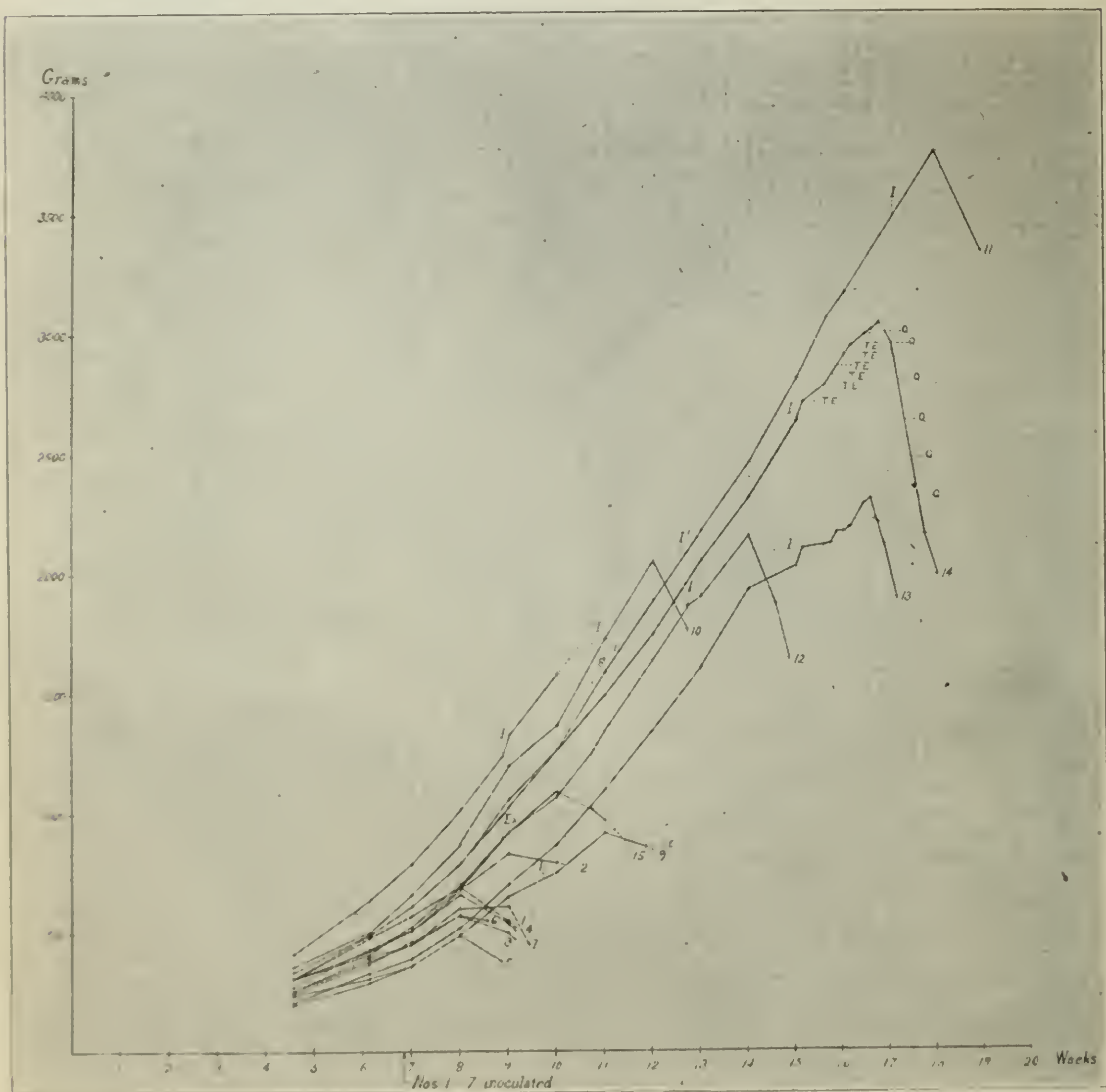

Chart 1. Numbers at ends of curves are numbers by which turkeys are designated. I., inoculation with blackhead virus; I.'. inoculation with sequestrum from chicken; Ex., exposure to respiratory discharges of inoculated turkeys. T. E., injection of tartar emetic; Q., injection of quinin dihydrochlorid.

far observed, occurred on the twelfth day, the latest on the twenty-fifth, the average being a little less than seventeen days.

The more important data of the various experiments on the inoculation and exposure of turkeys are given in table 1.

Virus						Weight, Gm.	
Inoculation 1	1	48 days 550 gm.	Subcutaneous	Marked weakness after 11th day	Died 14th day	170	Large local subcutaneous lesion. Secondary lesions in lungs and liver. Microscopically process older in lung than in liver
	2	48 days 520 gm.	Subcutaneous	Weakness about 17th day; cough 22d day	Dying. Killed on 25th day	40	Involvement of lungs extensive; liver shows 3 lesions and kidney one large lesion
Inoculated Aug. 14, 1919, with liver lesions of spontaneously infected turkey (C. P. S. 19.48)	3	48 days 460 gm.	Subcutaneous	No note	Died 16th day	100	Involvement of lungs extensive; liver, 2 minute lesions
	4	48 days 510 gm.	Subcutaneous	Developed peculiar gait on 11th day	Killed 16th day	150	Involvement of lungs extensive; one lesion against vertebral column. No nerve lesions found
	5	48 days 365 gm.	Subcutaneous	Extreme weakness on 14th day	Killed 14th day	110	Liver and ceca normal. Lungs not examined
	6	48 days 450 gm.	Subcutaneous	Dumpish on 11th day	Died 12th day	20	Liver and ceca normal. Lungs not examined
	7	48 days 450 gm.	Subcutaneous	Pale and dyspneic on 15th day; lively	Died 18th day	160	Involvement of lungs extensive; liver, 4 small lesions
Aug. 25, turkeys 1, 2, 3, 4, 5, 7 (coughing Aug. 27)	15	63 days 920 gm.	Exposed to sick inoculated turkeys	Weakness and sulphur colored droppings on 15th day	Died 22d day	120	Lesions in one of the ceca and in liver. Kidneys extensively involved (microscopically). Lung, minute microscopic lesion with organisms
Inoculation 2 Aug. 28 from turkey 5	8	62 days 1,240 gm.	Subcutaneous	Wings droop, sulphur colored droppings on 11th day	Died 15th day	No note	Death followed injection of tartar emetic. Involvement of lungs extensive. Liver, 4 small lesions
Sept. 3, from turkey 6570 (spontaneous case)	9	68 days 750 gm.	Subcutaneous	Weakness and coughing few days before death	Killed 15th day	60	Killed after injection of tartar emetic. Involvement of lungs extensive, other organs normal. Microscopically excessive reaction. Few remaining organisms isolated
Inoculation 3 Sept. 11, from turkey 8	10	70 days 1,740 gm.	Subcutaneous	Weakness on 11th day	Killed 13th day	290	Marked dyspnea. Involvement of lungs extensive; active process (microse). Liver, 2 small lesions
Inoculation 4 Sept. 24, from turkey 10	12	89 days 1,880 gm.	Subcutaneous	Pale and weak on 11th day; refused food	Died 15th day	520	Involvement of lungs. Liver normal. Kidney, one early lesion with organisms in large numbers
Inoculation 5 Oct. 9, from turkey 12	13	104 days 2,050 gm.	Subcutaneous	Weakness and yellow droppings on 13th day	Killed 16th day	420	Involvement of lungs extensive; diffuse pneumonia (microscopic chronic picture). Liver, several lesions; kidney, 2 lesions (extensive acute process microscopically)
	14	104 days 2,670 gm.	Subcutaneous	Pale, quiet on 14th day	Killed 23d day	1,070	Received injection of tartar emetic and quinin (see text). Large local lesion. Involvement of lungs extensive; necrosis of breast muscle at sites of inoculation
Sept. 23, from sequestrum from an inoculated chicken	11	88 days 2,200 gm.	Subcutaneous (chicken material)	None	No evident disease		Subcutaneous sequestrum persisted for weeks
Inoculation 6 Oct. 25, from turkey 13	11	120 days 3,590 gm.	Subcutaneous	Weak on 11th day; refused food	Killed 13th day	420	Local lesion small; involvement of lungs extensive; nodules and pneumonic areas (microscopically); liver 1 lesion (1 cm.)
Oct. 9 and 25 given flies fed on lesions	16	1½ mos. 740 gm.	Fed on "blow" flies	None	No evidence of disease		Made normal gain in weight
Nov. 9. Exposed to hens	16	2½ mos. 1,650 gm.	Exposed to hens	Weakness and yellow droppings on 22d day	Killed 24th day	330	Extensive involvement of right cecum. Liver, numerous depressed lesions (microscopically showing early infection)

* All turkeys in this column were 19: 19-1, 19-2, etc.

The inoculation of other species of birds and of laboratory animals with the virus of blackhead serves to demonstrate their relative non-susceptibility. Since blackhead occasionally occurs spontaneously in the common fowl,[2, 4] it might be expected that the subcutaneous inoculation of the virus would be attended with positive results in this species.

Chicken.—A series of 2-months-old White Plymouth Rock chickens, inoculated subcutaneously, intramuscularly, intravenously and intraperitoneally, respectively, failed to develop the disease. Several white Leghorns inoculated subcutaneously at the ages of 3½ to 4½ months, showed no definite local reaction and remained normal. The subcutaneous and intracecal inoculation of a number of Rhode Island Red chickens, some 2 and some 3½ months old, produced no evidence of disease. In such of the chickens as were killed or operated on, a flake of dry yellowish material was found at the site of inoculation. Such material, recovered 25 days from the time of inoculation of chicken (19-VI), and implanted in a turkey (19-11), failed to produce disease, indicating the death of the virus.

Four chickens, 48 hours after hatching, were inoculated with tissue of blackhead lesions. One of these chicks (19-4), showed a reaction at the site of inoculation 6 days later, and on the next day the lesion, appearing as a well defined, flat topped mass, was excised. It measured about 1 cm. across and 4 mm. in thickness, and was found to be composed of tough yellowish material surrounded by a ring of soft almost gelatinous pinkish tissue. (The inoculation of this material into 4 chicks of the same lot now 9 days old, produced in some, definite but transient reactions. See table 2.) The operation wound healed promptly, and there was no evidence of local recurrence of the disease. Sixteen days after the inoculation, the breathing was somewhat labored. The chick was killed, and pale grayish, rather indefinitely outlined lesions were found in both lungs. Histomonas meleagridis was demonstrated in fresh preparations from these foci. Of the other 3 chicks inoculated when 48 hours old, 1 showed a definite reaction after 7 days, and the other 2 after 9 days. Necrosis of the central portion of the lesions was apparent from the first, and this rapidly increased, forming a crust which involved the overlying skin. In one (chick 19-3) the process continued to extend up to the fourteenth day and formed a nodule of considerable size, but in the other two, regres-

[4] Milks, H. J.: Louisiana Agric. Exper. Station Bull., 1908, 108, p. 1.

sion followed soon after the development of the mass. In none of these was there evidence of involvement of internal organs. The transfer of the virus by the implantation of portions of the excised subcutaneous lesion of chick 19-4 into four 9-day-old chicks resulted in less well defined lesions. The subsequent inoculation of lung lesions obtained from chick 19-4 into 2 chicks 18 days old, resulted negatively.

Stained sections of the 6-day excised lesion of chick 19-4 showed acute inflammation with polymorphonuclear leukocytes in large numbers, but no necrosis. Moderate numbers of the parasite are present. The lung shows large foci of necrosis with extensive cellular infiltration, chiefly endothelial in type, and a few parasites. In general, the tissue reaction of the chicken to the virus seems to be of a more acute exudative character than in the turkey.

From the inoculation of chickens, it would appear that the virus develops for a time in very young chicks, but is soon destroyed by the reaction of the tissues. Involvement of the lungs occurred only in one from which the subcutaneous lesion had been excised. Even in slightly older chickens the virus fails to produce well defined active lesions. It was impossible to demonstrate the presence of virus in a 4 months chicken, 25 days after its inoculation, by the transference of the implanted material to the susceptible turkey.

Blackhead lesions were fed in large amounts to two 15-day-old chicks. The droppings of both these chicks were free from intestinal parasites and remained so on repeated examination. The microscopic inspection of the cecal contents and scrapings of the mucosa at necropsy, 5 and 8 days later, respectively, failed to show the presence of flagellates or other protozoa.

Table 2 gives the results obtained from the inoculation of very young chickens, but does not include the details of the experiments on older chickens, which invariably resulted negatively.

Pigeon.—The subcutaneous and intramuscular inoculation of pigeons has resulted in the production of a well marked but transient process in a certain proportion of cases, and slight or questionable reactions in several others. The virus was twice carried in pigeons to the second transfer, and once to the third transfer from the turkey. The failure to produce lesions on one occasion was evidently due to the employment of too old a lesion for inoculation purposes, for no organisms were distinguished in fresh preparations. and the lesions

had commenced to regress. In all, 29 pigeons were inoculated on 8 occasions. Nine produced well defined lesions, and the parasite of blackhead was demonstrated in fresh preparations from 5 of these. Several others showed slight or ill defined swelling about the implant. Parasites were most numerous in lesions taken on the seventh and eighth days after inoculation. They were found in one lesion on the tenth day, but were not demonstrable in two other lesions commencing to regress, one taken on the ninth and one on the tenth day.

TABLE 2

Results of Inoculation of Chickens

Date, Virus	Chicken	Age	Inoculation	Result	Remarks
Inoculation 1 Oct. 25: from turkey 13	1	48 hours	Subcutaneous	Local lesion; maximum development 10th day	Regression complete by 18th day; no secondary lesions
	2	48 hours	Subcutaneous	Local lesion; maximum development 11th day	Regression advanced by 14th day; no secondary lesions
	3	48 hours	Subcutaneuos	Local lesion; maximum development 14th day	Transformed to a crust; removed on 23d day, nearly healed
	4	48 hours	Subcutaneous	Local lesion; excised 7th day; active; numerous parasites	Wound healed rapidly; on 16th day labored breathing, killed; lesions in both lungs; parasites present
Inoculation 2 Nov. 1: from chicken 4	5	9 days	Subcutaneous	Small local lesion	Regression noted on 12th day
	6	9 days	Subcutaneous	No reaction	
	7	9 days	Subcutaneous	Well defined local lesion	Regression by the 12th day
	8	9 days	Subcutaneous	No definite reaction	Minute mass
Nov. 7 and 8: fed with lesions of turkey 11 and pigeon 18	9	15 days	Fed lesions	Negative	No flagellates appeared in feces
	10	15 days	Fed lesions	Negative	No flagellates appeared in feces
Inoculation 3 Nov. 10: from lung lesion, chicken 4	11	18 days	Subcutaneous	Small local lesion	Early regression
	12	18 days	Subcutaneous	Negative	

* All chickens in this column were 19: 19-1, 19-2, etc.

The local lesion develops at about the same rate in pigeons as in turkeys, but quickly regresses. Definite swelling, which appears around the implant 5 or 6 days after inoculation, increases rapidly for 2 to 4 days, and then quickly subsides. The process is thus at its height in 7 or 8 days, and is regressing 10 days after inoculation. The early 1-day lesion is found on section to consist of a small central sequestrum, representing the implanted tissue, surrounded by grayish opalescent tissue of soft consistence, evidently degenerated breast

muscle. More advanced lesions taken from 7 to 9 days after inoculation consist largely of firm, dry necrotic tissue, of dull pinkish color, more or less enclosed in a layer of opalescent grayish tissue which is not definitely demarcated from the surrounding muscle. In superficially situated lesions, the necrotic portion is apparent through the skin as a depressed central area, and this is surrounded by a ring of living tissue making the border of the lesion rounded and elevated. Regression soon follows and is attended with the subsidence of infiltration and the formation of sequestrum which becomes slowly absorbed. In no case did the disease spread to other parts of the body.

Microscopically, the inflammatory reaction is of a distinctly acute type and is even more pronounced than that observed in the newly hatched chick. In contrast with the reaction of the tissues of the turkey, purulent exudation is a prominent feature of the process. The parasites in the active 7-day lesion occur in a zone around the centrally situated necrotic tissue, and this in turn is surrounded by a thick layer of cellular infiltration. Peripheral to this between the separated muscle fibers are newly formed connective tissue and blood vessels. By the tenth day after inoculation, the parasites may have wholly disappeared and the lesion presents a sequestrum enclosed in a layer of lymphoid and endothelial cells.

It is thus possible to produce in pigeons a transient but well defined localized blackhead infection. About 30% of those inoculated developed active lesions and, if the cases are eliminated which were probably inoculated with nonvirulent material, the positive cases are raised to 39%. It is quite possible that blackhead might be transmitted in pigeons indefinitely by inoculation if active 7 or 8 day lesions were used for implantation, and a sufficiently large series of this species were employed. The abstracts of the experimental inoculations of pigeons are given in table 3.

Mammals.—Several species of mammals were also inoculated with the same material used in inoculating turkeys, chickens and pigeons. Rabbits were inoculated subcutaneously on the side and on the ear, intraperitoneally, and also in the testicle without success. A minute abscess which developed at the point of inoculation beneath the skin of the ear was excised 20 days after inoculation and sectioned, but no organisims were found. New-born guinea-pigs were inoculated subcutaneously and intraperitoneally with negative result. A small swelling, appearing in one case at the site of subcutaneous inoculation, was

excised and found to consist of necrotic material with no demonstrable parasites. Common mice and young Japanese waltzing mice were inoculated subcutaneously. Although small swellings appeared beneath the skin of the latter animals, stained sections showed only an acute inflammatory reaction to the foreign tissue and no parasites. Mammals have thus proved invariably nonsusceptible to the virus of blackhead.

TABLE 3

Results of Inoculation of Pigeons

Date, Virus	Pigeon*	Inoculation	Killed	Result	Remarks
Inoculation 1 Oct. 9, 1919, from turkey 12	1	Subcutaneous	5th day	+	Slight local infiltration
	2	Subcutaneous	8th day	++	Large local lesion; numerous parasites
	3	Subcutaneous	....	+	Transient reaction
	4	Subcutaneous	16th day	0	
Inoculation 2 Oct. 17, from pigeon 2	5	Intramuscular	....	0	Slight local reaction
	6	Intramuscular	10th day	++	Indurated m ss regression; no parasites
	7	Intramuscular	....	0	
	8	Intramuscular	....	0	
Inoculation 1a Oct. 25, from turkey 13	9	Intramuscular	7th day	++	Microscopically active lesion, many parasites
	10	Intramuscular	....	0	Indefinite swelling
	11	Intramuscular	9th day	0	
	12	Intramuscular	....	0	Indefinite swelling
Inoculation 3 Oct. 27, from pigeon 6	13	Intramuscular	....	0	Virus probably not present in material inoculated
	14	Intramuscular	....	0	
	15	Intramuscular	....	0	
	16	Intramuscular	....	0	
Inoculation 2a Nov. 1, from pigeon 9	17	Intramuscular	14th day	0	No definite local reaction
	18	Intramuscular	7th day	++	Large active local lesion; parasites present
	19	Intramuscular	14th day	0	Indefinite swelling on 11th day
	20	Intramuscular	14th day	0	No local reaction
Nov. 7, from lung lesion turkey 11	21	Subcutaneous	....	+	Definite swelling from 5th to 8th day
	22	Subcutaneous	8th day	++	Large lesion; parasites numerous
	23	Subcutaneous	10th day	++	Regressing
Inoculation 3a Nov. 8, from pigeon 18	24	Subcutaneous	....	++	
	25	....	9th day	++	Regressing
	26	....	....	++	
	17	....	....	0	
Nov. 10, from chicken 4	28	Intramuscular	....	0	
	29	....	....	0	

* All pigeons in this column were 19: 19-1, 19-2, etc.

THE INFECTIOUS CHARACTER OF INOCULATED BLACKHEAD

Prior to the discovery of lung lesions in the inoculated turkeys, it was assumed that the virus was confined to the nonulcerated subcutaneous lesions and was not discharged from the body. The 7 turkeys first inoculated were accordingly kept with the 8 normal ones. As

soon as it was determined that the lungs were involved, the question arose as to the possibility of the virus being discharged in mucus from the respiratory tract. That the disease may be transmitted spontaneously from inoculated to healthy turkeys is shown by the following data:

Of the brood of 15 young turkeys hatched on June 27, 1919, 8 were inoculated subcutaneously on Aug. 14, but were not isolated from the others of the flock. On Aug. 25, several of the inoculated birds were already showing weakness and loss of appetite, and within a few days there was more or less coughing among the inoculated birds, which continued until death. There was thus a possibility of contamination of the food and drink with the discharges from the respiratory tracts of the inoculated turkeys from Aug. 27 on. On Sept. 9, twelve days later, one of the uninoculated turkeys (19-15) passed sulphur colored droppings. The weight taken on the following day showed a loss of 70 gm. in the preceding 5 days. Death occurred on Sept. 16. The wall of one of the ceca presented several markedly thickened areas, ulcerated but without sequestrum or notable necrotic tissue. This cecum contained thick fluid of a reddish chocolate color. The other cecum appeared normal. The liver presented numerous characteristic lesions, many of which measured about 1 cm. in diameter. In the kidneys were several ill-defined, pale areas.

Stained sections of the diseased cecum showed extensive infiltration of the tissues with Histomonas meleagridis. There were no demonstrable flagellates in the cecal glands or on the surface of the mucosa. There were large numbers of Blastocystes in the cecal contents. Sections of the liver show characteristic lesions of blackhead. The kidney was extensively infiltrated with parasites. The involved areas were not well circumscribed. Some of these measure 5-8 mm. in diameter and appeared to be derived from the coalescence of smaller lesions from 1-2 mm. in diameter. Large numbers of the organisms were present, in association with extensive cellular infiltration but there was no necrosis. In the lung a minute microscopic lesion containing blackhead parasites was found.

Since this is the only case of spontaneous blackhead which occurred in the flock during the season, and since the first symptoms were noted 12 days after the appearance of coughing in the inoculated turkeys — an interval which coincides to a day with the average period of incubation in the inoculated disease and, as far as it has been ascertained, with the average incubation period of spontaneous blackhead — it appears most probable that this turkey became infected with the discharges from the respiratory tracts of the inoculated turkeys. That the contagion in this instance was derived from the lesions of the liver or kidney of the inoculated birds seems extremely unlikely, for not only were these lesions few in number, but organisms would probably be discharged from them much less readily than from

the infected lung. Furthermore, the probability of contamination of food and drink by respiratory discharges is much greater than by fecal material.

ATTEMPTED TRANSMISSION BY FLIES

Since young turkeys feed regularly on "blow flies" which collect on the droppings and especially on the cecal discharges, it was thought that these insects might act as distributors of the virus of blackhead. Flies of the species Calliphora erythrocephala Wied, were reared in captivity from eggs deposited on exposed strips of beef. The flies after emerging from the pupal cases were fed on sweetened water until used in this experiment.

On Sept. 15, a young turkey (19-16), about a month old but distinctly undersized, was obtained from a source where there had been more or less blackhead, although not in the brood from which this one was taken. This turkey was kept under observation in a brooder provided with a small wire enclosure wholly isolated from other turkeys and common fowls. Once or twice each day it was taken to an adjoining field to feed on grasshoppers and crickets. This turkey grew normally and showed no evidence of disease.

On Oct. 9, about 70 flies were fed with finely minced lung lesions and a portion of the subcutaneous lesion of an inoculated turkey, which were greedily devoured. Five hours later these flies were transferred to a clean cage in which they were transported about 15 miles, and were fed to the turkey 2 hours later. Sixty-eight of the flies were devoured and no other food except a few blades of grass were furnished until the next morning. There was no evidence of infection following this procedure which was again repeated on Oct. 25 when 67 "blackhead-fed" flies were devoured. The turkey remained normal and continued to grow rapidly for the next 15 days, indicating that the ingestion of the flies was evidently without effect.

EXPOSURE TO COMMON FOWLS

On Nov. 9 the turkey, which had been previously fed with infected flies, was placed with a small flock of hens on the same premises on which turkeys had been exposed to hens a year before. Twenty-two days later— on Dec. 1 — the first symptoms of blackhead appeared, and characteristic lesions of the ceca and liver were found on killing the bird on Dec. 3. Either the virus was present in this turkey for 37 days from the last feeding of flies without producing symptoms, or the disease was derived in the shorter period of exposure to hens. The latter explanation appears to be the more plausible, since the incubation period, in so far as it has been determined, appears to vary within comparatively narrow limits, i. e., between 11 and 17 days.

TREATMENT OF THE INOCULATED TURKEY WITH TARTAR EMETIC AND QUININ

In the summer of 1918 an unsuccessful attempt was made to prevent infection with blackhead by the administration of chaparro amargosa, a drug which has proved efficient in the treatment of amebic dysentery in man.[5] During the past season further experimentation along this line was undertaken, employing certain drugs known to be of value in the treatment of certain protozoan infections of the human being.

On Oct. 9, 1919, turkey 19-14, at the age of 104 days, was inoculated subcutaneously on the left breast with a bit of the subcutaneous lesion of another turkey (19-12), and on the right breast with a bit of similar material which had been kept frozen for 5 minutes previous to its implantation. The body weight on the following day was 2,670 gm.

On Oct. 11, 1 c c of a 1% aqueous solution of tartar emetic was injected into the wing vein. This was followed immediately by pronounced symptoms, i. e., panting, drooping of wings and unsteady gait—the latter persisting for about one hour.

The same dose of tartar emetic was injected on Oct. 14, 15 and 16, and 1.5 c c on Oct. 17, 18 and 20, more or less pronounced symptoms following each injection. In all, 85 mg. of this drug were injected. Swelling appeared around the implant on the left breast 5 days after its inoculation and a well defined lesion appeared which increased rapidly in size during the treatment with tartar emetic. Trichomonads, which had been noted in the cecal discharges previous to treatment, persisted in undiminished numbers. The body weight showed a steady increase, being 3,090 gm. on Oct. 22.

The first symptom of blackhead, i. e., failure to eat, appeared on Oct. 23, fourteen days after inoculation. The weight showed a drop to 3,050 gm. On this date, 1.5 cm. of a 10% solution of quinin hydrochlorid was injected, in part into the wing vein, in part into the breast muscle. On the following day the turkey developed a slight cough. On Oct. 24, 25, 26, 27 and 28, 1.5 cm. of the aqueous solution was injected, after which all treatment was discontinued. The total amount of quinin injected was about 900 mgm. The cough increased in frequency, and the disease showed steady progress during the treatment with quinin. After being carefully nursed for several days, the turkey appeared to be dying and was killed on Nov. 1, 23 days after inoculation.

Postmortem Examination.—Weight, 2,020 gm. (loss, 1,070 gm. from maximum weight attained); the primary lesion on the left breast measured 8 x 4 x 1.5 cm., and showed proportionately less necrotic material and a larger amount of pinkish translucent tissue than is usually seen in such lesions. Histomonas meleagridis was found in large numbers in fresh preparations. Posterior to the primary lesion was a pocket filled with chocolate colored fluid. The breast muscle at points where the injections were made showed in some instances glistening whitish areas, frequently several centimeters across. The lungs, with the exception of the apices, were for the most part replaced by large confluent lesions. One such lesion measured 4.5 x 4.5 x 3 cm. The left

[5] Tyzzer, E. E.: Jour. Med. Res., 1920, 41, p. 211.

lung weighed 45 gm. and the right 34 gm. In the mesentery were opaque masses of the size of a small pea, and on the surface of the left kidney were minute opaque grains about 0.5 c c in diameter. The liver, ceca and kidneys showed no evidence of involvement.

Microscopically the subcutaneous lesion showed a much thicker layer of reaction tissue infiltrated with organisms than had been observed in any of the other inoculated turkeys. The organisms were not only present in great numbers, but showed less evidence of degeneration than is usually encountered in the disease. The small lesions in the mesentery and on the surface of the kidney were not characteristic of blackhead, and may have resulted from the injection of one or the other of the drugs employed.

It is apparent from these results that neither tartar emetic nor quinin is of any therapeutic value in blackhead. The primary lesion showed, if anything, greater development than usual, and the course of the disease was not appreciably modified. The implantation of a bit of lesion which had been frozen for five minutes failed to produce an active lesion.

DISCUSSION

The experiments here outlined demonstrate the uniform susceptibility of turkeys to blackhead. The regularity of the inoculated disease irrespective of weather conditions or the size, and as far as has been determined, the age of the turkey, indicates that all that is necessary for the production of the disease is the entrance of the parasite into the tissues. Whether conditions which are supposed to lower the resistance of these birds, or functional disturbances of any kind, which are possibly more frequent in early life, tend to favor the entrance of the parasite is not known. It appears possible, from what has been learned of the nonresistant properties of the latter outside of the body and of its behavior when introduced into the tissue, that those conditions which are supposed to lower the resistance of the turkey in reality are less destructive to the parasite and so favor its transmission.

Of the other species employed in these experiments, none approach the turkey with respect to susceptibility. Since ruffed grouse reared in captivity commonly succumb to this disease, it is quite probable that this species may be as susceptible as the turkey. The common quail is also somewhat susceptible. It is rather remarkable that the common fowl should prove so refractory to the infection, especially as spontaneous cases have been occasionally observed in this species. It is difficult to account for such cases — possibly certain breeds or stocks are more susceptible; their resistance may be lowered by unfavorable conditions; or more virulent or adapted strains of the blackhead para-

site may be present. The disease has been produced by the inoculation of young chickens and, although in most cases it was self-limited, in one case it involved the lungs. A transient local infection in pigeons may be produced by inoculation, but the mammals inoculated have proved invariably nonsusceptible.

The virus employed has remained remarkably constant, the incubation period has not altered, and there has been no diminution of virulence in the course of these experiments.

The conclusion reached by Hadley[6] that blackhead is caused by the invasion of the tissues with an intestinal flagellate, Trichomonas, species undetermined, lacks satisfactory proof. From his descriptions and illustrations it is apparent that he had under consideration a flagellate of the genus Trichomonas (figs. 1 and 2), an organism of the Blastocystis type (figs. 11, 12 and 29), and the tissue parasite which had been previously described by Smith as the causal agent of blackhead. These are regarded, on the basis of transitional forms and of topographical relationship to goblet cells or breaks in the epithelium, as developmental stages of a single species. The flagellated forms are said to measure from 8-12 microns in length, and to possess three anteriorly, and one posteriorly, extending flagella, an undulating membrane, axostyle, chromatic line, chromatin blocks and other features, all of which correspond rather closely with the description of Trichomonas eberthi Martin and Robertson. Multiplicative processes are described by this author, however, which are without analogy in other species of Trichomonas, so that it does not appear unreasonable to question whether he has not mistaken several mingled species for developmental forms of a single species.

In the present study of a large series of cases of blackhead no morphologic evidence has been found suggesting the transformation of the associated cecal trichomonad into the organism of blackhead. Although the latter parasite is found contiguous to, and occasionally on, free surfaces, forms of it have never been observed which would suggest its identity with Trichomonas eberthi which occurs in the cecal glands. In the study of stained sections of the infected lungs, an occasional example of Histomonas meleagridis was observed in which the blepharoplast and radiating filaments were situated at the surface of the cytoplasm at a distance from the nucleus, but such forms were not only morphologically unlike any of the flagellates thus far found

[6] Agric. Exper. Station, Rhode Island State College Bulls. 166 and 168, 1916.

in the cecal glands, but were also much larger. Although the movements of the blackhead parasite as observed in the warm chamber as well as its morphology, indicate a close relationship to the trichomonads, no evidence of its identity with any of the established species of the latter is at present available. The study of examples of the parasite which have passed from the tissues into the air spaces of the lung, the bile ducts, and the urinary tubules, has thus far revealed none which has assumed the morphology of the flagellated trichomonad. It has not yet been demonstrated that this parasite can multiply in the lumen of the intestine or elsewhere outside the tissues. Young chickens free from intestinal protozoa were fed large amounts of blackhead virus, with a view to obtaining a flagellated stage of the parasite in the intestine. Entirely negative results followed, no flagellates or other protozoa being demonstrable during life or on postmortem examination of the ceca.

Although Hadley claims that there are transitional stages between the flagellated Trichomonas of the cecal glands and the tissue parasites, he has furnished no satisfactory evidence of such transformation. The presence of numerous flagellates beneath the epithelium, without associated tissue reaction, may only be accounted for by postmortem invasion or by artefact in the process of preservation. Their occasional occurrence in diseased or necrotic tissue may be accounted for by secondary invasion. Under these circumstances they are promptly taken up by phagocytes, but instead of assuming the appearance of Histomonas, retain their characteristic trichomonad morphology. The presence of Blastocystis beneath the surface of the cecal mucosa [7] indicates that cecal contents have in some way been forced into the tissue beneath the dislodged epithelium.

Invasion of the tissue is said by him to be preceded by excessive multiplication of the flagellates and "the swarming of the motile stages from the cecal content into the crypts, in such numbers as to menace the integrity of the epithelial wall." The absence of flagellated organisms from the ceca in certain acute cases of blackhead does not conform with this view. In turkeys 19-15 and 19-16, of the present series, no flagellate was demonstrable in either the cecal contents or the glands. Furthermore, the appearance of great numbers of trichomonads in the cecal discharges of turkeys reared at the Medical School in the summer of 1918, showed no significant time relationship to the occurrence of blackhead in this flock. Cases of the disease appeared,

[7] Hadley, P. B.: Agric. Exper. Station, Rhode Island, Bull. 168, Fig. 29.

however, after exposure to infected turkeys and to common fowls, and the time from exposure to the appearance of symptoms is consistent with what is known of the period of incubation in blackhead.

In his consideration of the multiplication of the flagellates in the ceca, Hadley discusses "the typical flagellate dysentery, which is almost invariably the precursor of blackhead." Of the cases of blackhead which have occurred in turkeys reared here under observation during the summers of 1918 and 1919, none has shown diarrhea previous to the onset of the disease. The first symptoms consisted of weakness, loss of appetite, and the appearance of sulphur colored droppings. In young growing birds soft droppings are not abnormal, and the passage of soft, semifluid discharges from the ceca do not indicate dysentery.

By experimental introduction of the parasite into the subcutaneous tissue instead of into the cecal wall, as in spontaneous infection, a disease, inoculated histomoniasis, has been produced, which presents distinct differences from the natural infection. These differences are dependent on the distribution of the lesions — while in spontaneous blackhead the primary lesions are situated in the ceca and the secondary lesions in the liver — in the inoculated disease with the primary lesion beneath the skin, secondary lesions occur constantly in the lungs and occasionally in other organs, and smaller tertiary lesions may occur in the liver and kidney. The distribution of lesions apparently does not depend on the restriction of the growth of the parasite to certain tissues or organs, for as far as it has been ascertained it multiplies readily in any of the soft tissues. Taking the invasive properties of the parasite into account in both the natural and the inoculated disease, the difference in the distribution of lesions is clearly attributable to the relation of the primary lesion to the vascular system.

Thus in the course of the migration of the parasites through the tissues of the cecal wall in spontaneous blackhead, a certain number penetrate the portal veins and are transported to the liver. It is evident that this organ usually serves as an effective filter for the parasites, as lesions have not been reported in the lungs in the spontaneous disease. However, in recently studied cases of spontaneous blackhead, the authors have demonstrated that the organism, after lodging in the liver may subsequently be disseminated to other organs. Early lesions of the kidney with numerous parasites, and in one case a microscopic lesion of the lung containing parasites, have been found in the natural disease.

In the inoculated disease, the parasites multiplying beneath the skin, may penetrate the walls of the systemic veins and thence be carried to the lungs, where they lodge to produce secondary lesions. The lung, however, does not constitute such an effective barrier as the liver to the passage of the parasite, for lesions of the liver and kidney (see fig. 4) are occasionally encountered, which have evidently developed simultaneously with those of the lung. In most cases the lesions of the liver and kidney are of relatively small size as compared with the older lesions of the lung, and may thus be designated as tertiary in character. It appears quite probable that the movements of the lung in alternate expansion and contraction are more favorable to the passage of the parasite through its capillaries than is the case in the liver. The usual failure of the parasite to pass through the sinusoids of the liver makes improbable the occurrence of the hypothetical small forms suggested by Theobald Smith. Its dissemination is evidently quite analagous to that of the cells of certain transplantable, metastasizing tumors of rats and mice, and appears to be wholly through the blood vessels. These facts are consistent with the size and physical characteristics of the known forms of the parasite.

The initial dissemination of the organisms by the blood stream is evidently coincident with the infiltration of the tissue around the subcutaneous implant, which appears from 5 to 7 days after inoculation. It appears reasonable to assume that the size of the lesion is proportionate, at least during the acute phase of the disease, to the number of organisms present. By allowing an equal period of time for the development of lesions in the lung and beneath the skin, dissemination of the parasites by the blood stream would be initiated as early as the middle of the period of incubation, that is, at the time of the appearance of a definite local reaction. The rapid macroscopic changes which take place from 5 to 7 days after inoculation are quite in accord with the multiplication of the parasite by binary division, a point determined by histologic studies.[2] It has already been pointed out by Smith that the initial reaction of the tissues amount to little more than distention by innumerable parasites. Great numbers of the latter evidently perish from the effects of overcrowding during the acute phase of the process; others survive to become phagocytosed by the tissue cells that eventually assume the form of giant cells.

There is no definite evidence of the production of markedly toxic substances by the parasite. Rectal temperatures taken 9 and 15 days

after inoculation showed no definite variation from the normal, as may be seen in table 4.

TABLE 4

Rectal Temeratures 9 and 15 Days after Inoculation

	Eight Control Turkeys			Seven Inoculated Turkeys		
	Maximum	Minimum	Average	Maximum	Minimum	Average
August 23	107.9 F.	106.0 F.	106.69 F,	108.2 F.	105.8 F.	107.2 F.
August 29*	107.9 F.	106.5 F.	107.26 F.	107.9 F.	105.3 F.	107.1 F.

* Only five inoculated turkeys survived to this date.

The rapid extension of the subcutaneous lesion is unattended by symptoms, and it is only after vital organs have become seriously diseased that the turkey appears ill or loses weight. It is thus conceivable that spontaneous blackhead might in some instances be arrested without serious impairment of functions, so that cases may occur without symptoms. No evidence of such unrecognized attacks has been obtained in the present investigation, for all the turkeys employed proved to be susceptible to the disease. Neither is there anything to indicate latency of infection, since the incubation period in the entire series showed no great degree of variation.

In this infection death appears to be due to the extensive involvement of vital organs: the lung and less frequently, the liver and kidney in the inoculated disease, and the ceca and liver in the spontaneous disease. Terminal secondary infection evidently occurs for bacteria are frequently obtained in cultures from the liver.

The pathology of the disease, especially with reference to the distribution of the parasites in the body, has a distinct bearing on the question of its transmission. In spontaneous blackhead the virus is largely confined to the ceca, the portal veins and liver. The natural outlet for it, therefore, appears to be the cecal discharges or the bile and thence the feces. In case the kidney is also involved the virus may be discharged in the urine. There is thus slight possibility of its being transmitted by a biting insect, for the virus is rarely present in the circulation outside the portal veins.

In the inoculated disease, on the other hand, the virus passes from the local lesion through certain of the systemic veins, to the lungs, and to a less extent to the liver, and occasionally to the kidney. In this

form of the infection, the chances of its escape through the feces appear slight, as compared with the probability of its discharge with mucus in coughing. The parasite is not discharged in enormous numbers in either form of the disease. They are occasionally found in stained sections within giant cells free on the surface of the cecal mucosa and in the lumina of bile ducts, but have not been identified in the discharges during life. They have been observed on one occasion in mucus from the larynx, but are evidently rare. Stained sections of the lung in the inoculated disease usually show, however, great numbers of cells containing parasites free in the air spaces, but rarely more than an occasional free trophozoite. It is evident that active forms of the parasite seldom leave the tissues, but that quiescent forms may be discharged within the cells which have effected their isolation. It is not definitely determined that the forms, previously interpreted as representing a resistant phase of development, are capable of multiplying when transferred to normal turkeys, although it is quite evident that a large proportion of the organisms are destroyed by the giant cells which appear within the lesions.

Various possibilities may be taken into consideration with respect to the transference of the virus from the disease to normal birds. It is not known at present how long the parasite will remain alive outside of the body. From what has been observed of its characteristics, it appears to be an extremely frail, nonresistant organism, and there is no evidence for the assumption that it lives from one season to another in the soil. Although blackhead was introduced into the yard at the Medical School in the summer of 1918, fifteen turkeys were raised in confinement on the same soil during the following season, without the appearance of any case that could be attributed definitely to this source.

The louse evidently plays no rôle in the transmission of the disease, for not only is Histomonas rarely present in the peripheral circulation, but also the case of spontaneous disease recorded earlier in this paper occurred in an incubator hatched turkey on which there were no lice.

Flies, especially the "blow flies" which feed on the cecal discharges, should be considered as possible agents in the distribution of the parasite, although failure resulted in the single instance in which the transmission through flies was attempted experimentally. These flies feed constantly on the discharges during fair weather and are caught

regularly by young turkeys. If the virus is able to survive even for a short time in or on these insects, their movement from place to place would distribute it.

In a recent paper by Graybill and Theobald Smith,[8] attention has been called to the importance of a species of intestinal worm as a factor in the transmission of blackhead in turkeys. Since the present investigation has been chiefly confined to the study of the inoculated disease, no conclusive evidence has been obtained relative to this interesting observation.

With the production of the disease by inoculation, we now have a means of studying more carefully the properties of the virus, its resistance to variation of temperature, to drying, to chemicals, etc. It is now possible to ascertain the effects on the parasite of various drugs administered to the infected host, and to attempt to modify the virus. It may also prove possible to demonstrate the virus in birds suspected of being carriers.

SUMMARY AND CONCLUSIONS

A distinct form of blackhead may be produced in turkeys by the subcutaneous inoculation of liver lesions from acute cases, and this disease may be propagated apparently indefinitely, by subinoculation into normal turkeys. This form of the disease is characterized by the appearance of a primary local lesion which first shows appreciable development from 5 to 7 days after inoculation; by the occurrence of secondary lesions regularly in the lungs, rarely in the liver and kidney, and by the later involvement of the liver and kidney (tertiary lesions) through the dissemination of the parasite from the involved lungs. In addition to weakness, loss of appetite, and sulphur colored feces—symptoms which are seen in the spontaneous disease—there is coughing and more or less dyspnea. The inoculated disease has been invariably fatal.

The incubation period is commonly 11 days, but varies between 11 and 17 days. The appearance of symptoms evidently indicates sufficient involvement of vital organs to interfere seriously with function. The rapid development of the subcutaneous lesion is not attended either by loss of weight or by symptoms, neither of which appears until after internal organs are involved.

In the course of their migration through the tissues from the site of inoculation, some of the parasites penetrate the veins and are

[8] Jour. Exper. Med. 1920, 31, p. 647.

carried to the lungs where they for the most part lodge and produce lesions. The disease thus metastasizes by way of the blood stream in a manner similar to that of certain tumors. The distribution of the parasites is thus governed by their ability to penetrate vessels, and by their size and physical properties which cause them to lodge in capillaries.

The expansion and contraction of the lungs evidently serve to dislodge organisms so that these organs are not as effective filters as the liver.

The parasites develop readily in a variety of tissues and organs: mucous membranes, connective tissues, both smooth and striated muscle, lung, liver and kidney. Macroscopic lesions of the kidney and microscopic lesions of the lung have been found in spontaneous blackhead.

The inoculation of chickens has resulted negatively except in newly hatched chicks in which self-limited local lesions, and in one instance, secondary lesions in the lungs, were produced.

A certain proportion of pigeons have proved susceptible to the extent of developing transient self-limited local lesions. Positive results were obtained in 30% of those inoculated. The lesions are essentially similar to the subcutaneous lesions of the turkey, but after developing actively from the fifth to the eighth day undergo regression. Rabbits, guinea-pigs and mice have proved nonsusceptible.

Blackhead may be contracted spontaneously from acute cases of its inoculated form, probably from the ingestion of food or water contaminated by discharges from the respiratory tract.

An attempt to transmit the disease through the agency of a species of "blow fly" has failed, but it is possible that this or related species may play a part in the dissemination of the disease.

Exposure of a young turkey to common fowls, after a long period of isolation, has been followed by the contraction of blackhead—unless the infection is to be attributed to the "blackhead-fed" flies ingested by it 37 days previously.

Neither the administration of tartar emetic nor of quinin has served to check the course of the infection.

Blackhead may be produced by the introduction of organisms beneath the skin, and is not dependent on the invasion of the tissues by myriads of flagellates that have multiplied in the lumen of the gut.

No flagellates have appeared in the ceca of newly hatched chickens following the ingestion of large amounts of blackhead virus derived from subcutaneous and lung lesions.

Cases of spontaneous blackhead occur in which there are no demonstrable flagellates in the cecal contents or in the gland lumina.

The definite transmission of the disease from one turkey to another by inoculation at once removes the great uncertainty which has attended all experiments in the past which have had to depend on the natural and not well understood method of transmission by the exposure of healthy turkeys to supposedly infected turkeys.

The demonstration of the uniform susceptibility of the normal turkey throughout its period of growth, not only opens the way for further investigation of the question of transmission, but also serves as a basis for future work on other problems relating to the virus of blackhead.

ADVANTAGES OF SOLID PARAFFIN FOR SEALING ANAEROBIC FLUID CULTURES

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In 1901, Park[1] recommended the use of solid paraffin for sealing anaerobic fluid cultures. Solid paraffin has been used subsequently by Weinzirl,[2] but apparently has not been adopted by other workers. It was the object of the present study to test the advantages of solid paraffin seals over the ordinary liquid paraffin method, and to devise a simplified solid paraffin technic.

TECHNIC

Preparation of Culture Tubes.—Paraffin melting at about 55 C. is heated well above the melting point, and delivered, in measured quantities, by means of a flame-heated pipet, into dry test tubes. With the ordinary half-inch test tube, 0.5 c c paraffin is sufficient. The tubes are plugged and sterilized in the upright position in an autoclave. After sterilization, the paraffin is allowed to solidify at the bottom of the tubes.

The liquid culture medium is now added to the tubes, and the tubes sterilized in an upright position by the discontinuous method. After each heating, the tubes are rapidly cooled by immersing them in cold water. After the final heating, the thoroughly cooled medium is stored until needed. The paraffin at this stage forms a solid layer from 2 to 5 mm. thick, at the top of each tube (fig. 1, A).

Inoculation of the Tubes.—The tube is gently warmed over a Bunsen flame, at the level of the paraffin plug. The plug can then be readily tipped with the platinum loop (fig. 1, B). After inoculation, the tube is again warmed slightly as before to seal the plug, and placed in an upright position in cold water for a few seconds.

Cleaning the Tubes.—To remove the paraffin after the tubes have been used, the cotton plugs are withdrawn, the tubes completely immersed in an upright position in water, and heated in a steam sterilizer. During the heating, the paraffin rises to the top of the water, from which it can readily be removed as a solid cake, after cooling.

CULTURAL ADVANTAGES

To test the advantages of the solid paraffin seal over the ordinary liquid paraffin method, parallel inoculations were made with stock anaerobic cultures, by the two methods. It was found that a tur-

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[1] Jour. Boston Soc. Med. Sci., 1901, 5, p. 373.

[2] Science, 1915, 42, p. 353.

bidity usually develops under the solid paraffin seal, sooner than under liquid paraffin. A comparison of this feature is shown in Figure 2, in which the inoculated tubes have been photographed against a wire screen to show relative turbidity.

It was further found that the percentages of positive cultures obtained with the tubes sealed with solid paraffin is greater than the percentage in the parallel inoculations by the liquid paraffin method.

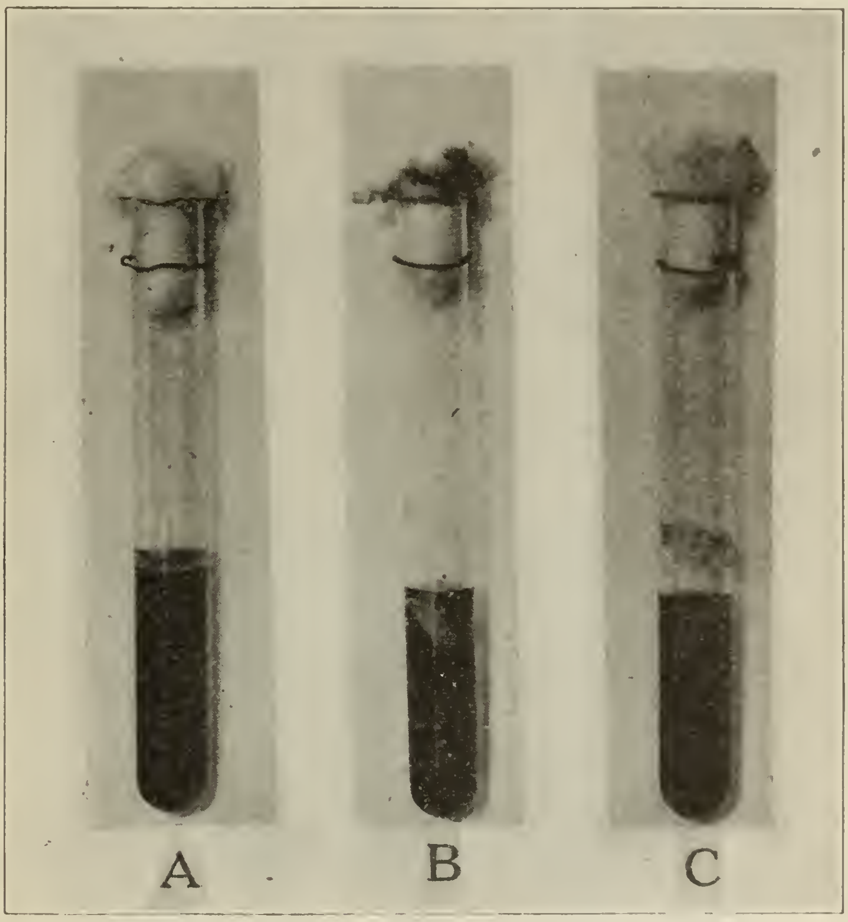

Fig. 1.—Solid paraffin seals. A, culture tube before and after inoculation. B, paraffin seal tipped during process of inoculation. C, paraffin seal lifted by gas formation.

This comparison is shown in table 1, in which 100% positive takes are recorded for the solid paraffin method, as contrasted with but 54% positive takes with the liquid paraffin method.

EXPLANATION OF ADVANTAGES

To determine the reasons for this advantage, tests were made of the relative rates of oxidation of colorless (heat reduced) litmus solution, by the two methods. Data from such tests are shown in figure 3.

From these data it is seen that slowly cooled, uncovered culture medium is oxidized within three hours, the oxygen entering the

TABLE 1

Comparison of Solid Paraffin and Liquid Paraffin Methods

Organism	Solid Paraffin			Liquid Paraffin		
	Inoculations	Growth	Percentages	Inoculations	Growth	Percentages
B. edematis.....	27	27	100	26	20	77
B. welchii.......	16	16	100	18	6	33
B. botulinus....	15	15	100	18	11	61
B. tetani........	16	16	100	18	6	33
Total........	74	74	**100**	80	43	**54**

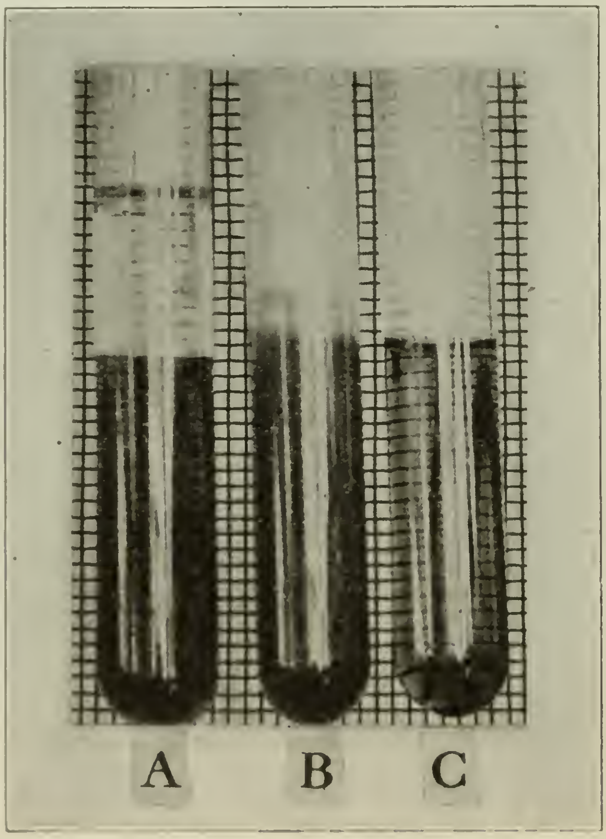

Fig. 2.—Comparative rapidity of growth under solid paraffin and liquid paraffin seals, ten hour stage, B. edematis. The tubes have been photographed against a wire mesh to show relative turbidity. A, culture under liquid paraffin seal, showing beginning turbidity in the lower portions of the tube. B, culture under solid paraffin seal, showing marked turbidity in the entire tube. C, aerobic control, showing no turbidity.

medium largely by downward convection currents during the process of cooling.

If uncovered medium is rapidly cooled, the entrance of oxygen is slower, the lower portions of the tubes remaining colorless for two or

three days. Anaerobes will occasionally grow in rapidly cooled tubes, without a protecting layer of paraffin. A similar observation has been made by others.[3]

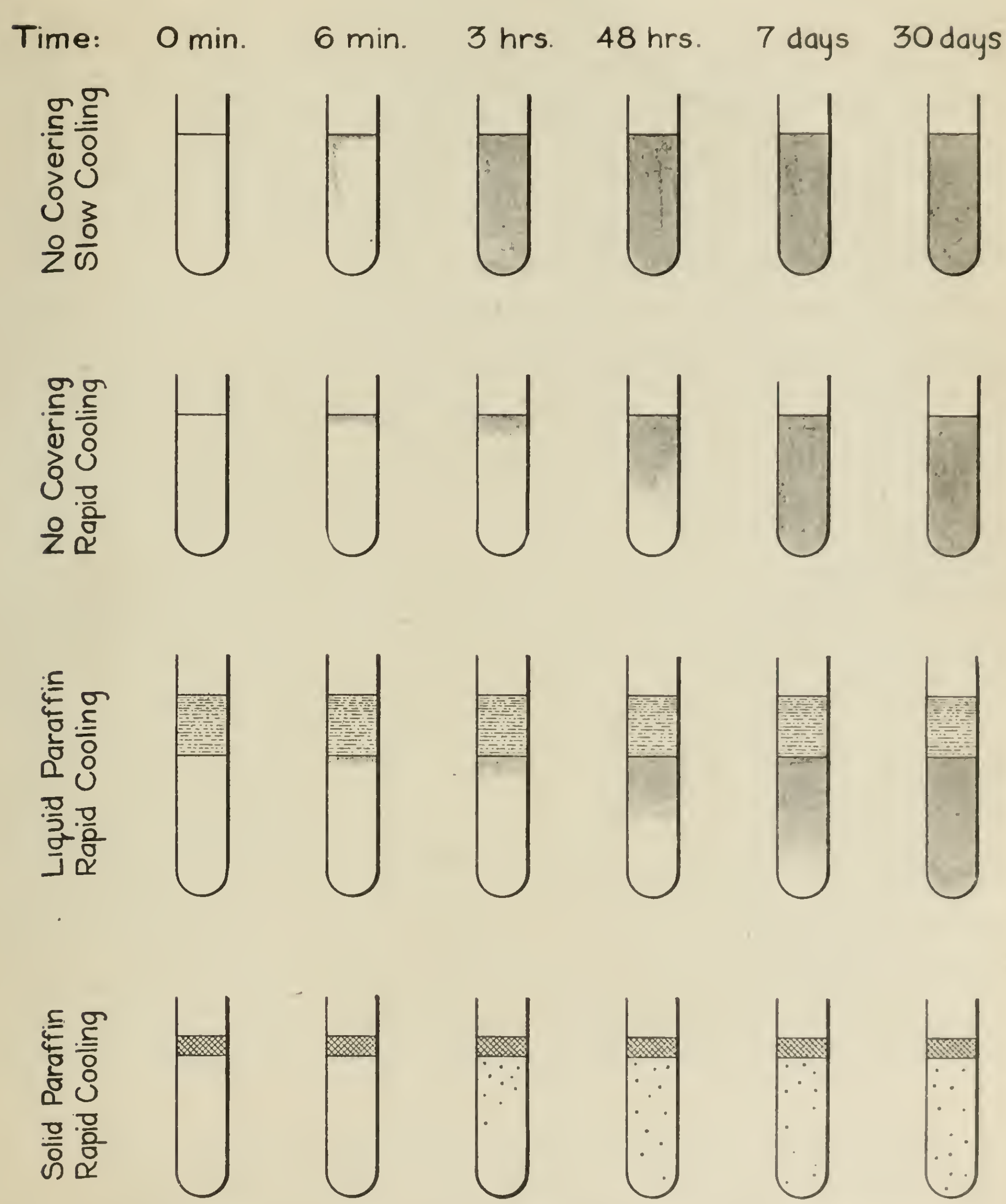

Fig. 3.—Relative rates of oxidation of heat reduced litmus. Shading shows distribution and relative intensity of the blue tint. In the solid paraffin tubes, a slight blue tint appears near the surface, during the few minutes before the paraffin becomes solidified. After solidification, there is no appreciable increase in the blue tint, even in tubes stored for 30 days. Handling, shaking and changes of temperature do not increase the rate of oxidation in these tubes.

If the rapidly cooled tubes are covered with liquid paraffin, they become oxidized much more slowly, the deeper portions of the tubes

[3] Rosenthal, C.: Compt. rend. Soc. Biol., 1906, 61, p. 440.

remaining colorless for from one to two weeks. If, however, these tubes are handled, shaken, or subjected to changes in temperature so as to set up convection currents, they may become blue within a few hours.

Rapidly cooled tubes, sealed with solid paraffin, take on a slight bluish tint near the surface, during the few minutes before the paraffin is solidified. After solidification there is no appreciable increase in the blue tint, even at the end of thirty days. Shaking, handling, or ordinary changes in temperature have no appreciable effects on the entrance of oxygen into these tubes.

SUMMARY

Tubes sealed with solid paraffin, by the method described, give a greater percentage of positive growths with stock anaerobic cultures than parallel tubes sealed with liquid paraffin.

This advantage is due to the prevention of the downward diffusion of oxygen by convection currents in the tubes sealed by the solid paraffin method.

PATHOGENIC BACTERIA IN HOG CHOLERA BLOOD

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A great deal of interest is centered about the question of the importance of pathogenic secondary invading bacteria in hog cholera blood. This question is of practical interest on account of the possible causal relationship of these secondary invaders to the losses which frequently occur in hogs injected with large amounts of hog cholera blood in the production of antihog cholera serum. In this study it is attempted to determine the relationship between the presence of gas-producing bacteria in hog cholera blood and the pathogenicity of such blood for rabbits and hogs.

Gas Production and Pathogenicity Tests.—One c c of each sample of hog cholera blood was inoculated into fermentation tubes of pork infusion broth containing 1% of glucose. The inoculated tubes were then incubated for 3 days, with observation on each day for gas production. Rabbits were inoculated subcutaneously with 1 to 2 c c of cholera blood. Hogs were injected intravenously with 4 to 6 c c of hog cholera blood per pound body weight.

The hog cholera blood was obtained from cholera-susceptible pigs 5 to 9 days after the pigs had been injected with hog cholera blood for the purpose of propagating the hog cholera virus. Table 1 shows the percentage of gas-producing samples obtained by bleeding on different days after inoculation.

The inoculation of 15 rabbits with gas-producing samples of hog cholera blood resulted in the death of 10, while of 51 rabbits inoculated with nongas-producing blood only one died. With one exception, the deaths of these rabbits occurred 7 to 12 days subsequent to inoculation. This single exception occurred in the case of the one rabbit that died following inoculation with nongas-producing blood. This rabbit died on the third day after inoculation, and an organism morphologically and culturally B. suisepticus-like was isolated from the heart blood.

The data in table 1 indicate that, after the fifth day following inoculation of hogs with cholera virus, there was a marked increase in the proportion of cholera-infected hogs having gas-producing bacteria in their blood. This proportion was much higher on the eighth and ninth days than on the sixth and seventh days. The results of inoculations of rabbits show a close relationship between the presence of gas-producing organisms in hog cholera blood and the pathogenicity of such

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blood for rabbits. It appeared that, with one exception, hog cholera blood which did not contain organisms capable of fermenting glucose with the production of gas was harmless for rabbits when inoculated subcutaneously. This single exception, in 51 cases, was one in which a B. suisepticus-like organism was isolated from the heart blood of a rabbit that died 3 days after inoculation with nongas-producing cholera blood.

TABLE 1

Relation of Percentage of Gas-Producing Samples of Hog Cholera Blood to Time of Bleeding

Samples	Day of Bleeding	Percentage Producing Gas
20	5-day	0.0
79	6-day	6.7
162	7-day	6.8
14	8-day	14.2
15	9-day	13.3
566	Unclassified*	12.0

* This group consisted of 7, 8 and 9-day virus.

While the hog cholera blood containing gas-producing organisms appears to be virulent to a high degree for rabbits, the same blood may be harmless for hogs, even when injected intravenously in large quantities. Thus the common assumption of correlation of the pathogenicity of such organisms for rabbits and swine does not appear to be sustained. Of 72 hogs injected with cholera blood containing gas-producing bacteria, only one died; whereas, of 175 hogs injected with cholera blood which did not contain gas-producing bacteria, 8 died. While these hogs had previously been vaccinated and were presumably immune, it is quite possible that they did not possess a degree of immunity sufficient to withstand the large dose of hog cholera virus. Susceptibility in presumably immune hogs has been demonstrated on other occasions, and, granting such susceptibility in the presence of the filterable hog cholera virus, the presence or absence of bacteria probably has no material influence on the course of the disease.

Bacteriologic Examination.—Since the results of rabbit inoculation with hog cholera blood indicated a close relationship between the presence of gas-producing bacteria and pathogenicity of such blood for rabbits, a qualitative bacteriologic examination was made of a number of samples of cholera blood. As this study has been reported in part in a previous publication,[1] only a brief outline of the technic employed will be given.

[1] Spray, R. S.: Jour. Infec. Dis., 1920, 26, p. 340.

Cultures were isolated by dilution plating from glucose broth fermentation tubes or by direct plating of the hog cholera blood. Cultures were isolated from rabbits by plating heart blood of animals dying following inoculation with hog cholera blood. These cultures were replated several times to insure purity, and stock cultures were picked from typical well isolated colonies. Preliminary and presumptive paratyphoid tests were made on these stock cultures, and cultures not conforming to the characteristics of the paratyphoid-enteritidis group were discarded. Having satisfied the presumptive tests, the cultures were put through a series of cultural tests, the results of which are recorded in table 2.

TABLE 2

Essential Diagnostic Characteristics

Cultures	Glucose Serum Water	Lead Acetate Agar	Milk Alkaline at	Translucent Opalescent at 90 Days	Arabinose Serum Water	Xylose Serum Water	Dulcite Serum Water	Inosite Serum Water
B. suipestifer type Strain 49	+	—	4th day	+	——	++	——	——
Virus 108, 125, 127, 138, 145, 146, 161, 167, 187, 192, 193	+	—	8-10 days	+	——	++	——	——
Rabbit 107*	+	—	8th day	+	——	++	——	——
B. paratyphosus A, Human A98	—	+	38th day	—	+—	——	++	——
Human A97, Rabbit 134*, Virus 149	+	—	35-38 days	—	+—	——	++	——
B. paratyphosus B, Human B96, Rabbit 124, 135*	—	+	3-4 days	+	++	++	++	++
Intermediate, Rabbit 91*, Virus 201, 202	—	+	6-7 days	+	——	++	——	——
B. enteritidis, Human E117	—	+	2d day	+	++	++	++	——

* Strains isolated from rabbits inoculated with virus blood known to contain gas-producing bacteria.

Table 3 indicates that, while 12 strains of typical B. suipestifer were isolated, other paratyphoids may frequently be encountered in the study of hog cholera blood. Two strains each of organisms identical with B. paratyphosus A and B strains of human origin and three interesting intermediates were isolated from cholera blood or from rabbits dying after inoculation with cholera blood. In the study of another disease of swine [1] on two occasions intermediate strains, identical with those isolated from cholera blood and rabbits, were also isolated from the spleen. The significance of the presence, and the relationships of these intermediates are uncertain, but they apparently constitute a well-defined and constant type which may be frequently encountered in the study of pathologic swine tissue.

In order to compare and study these intermediates further, cross-agglutination tests were made with authentic strains of B. paratyphosus A and B, B. enteritidis, and B. suipestifer.

TABLE 3

Highest Serum Dilution Showing Perceptible Agglutination

Cultures	Serum				
	B. suipestifer 49	Intermediate 91	B. paratyphosus B96	B. paratyphosus A98	B. enteritidis E117
B. suipestifer type, Strain 49	1:30000	1:15000	1:3000	....	....
Virus 108, 125, 127, 138, 145, 146, 161, 167, 187, 192, 193	1:30000	1:15000	1:3000	....	....
Rabbit 107	1:30000	1:15000	1:3000	....	....
B. paratyphosus A. Human A98	....	....	....	1:15000	....
Human A97, Rabbit 134, Virus 149	....	....	....	1:15000	....
B. paratyphosus B, Human A96, Rabbit 124, 135	1:3000	1:3000	1:15000	....	....
Intermediate, Rabbit 91, Virus 201, 202	1:15000	1:30000	1:7500	....	....
B. enteritidis, Human E117	....	....	....	....	1:7500

TABLE 4

Absorption Tests Showing Relationship Between Intermediates, B. Suipestifer and B. Paratyphosus B

Cultures	Serums								
	B. suipestifer 49			Intermediate 91			B. paratyphosus B96		
	Absorbed by Intermediate 91	Absorbed by B. paratyphosus B 135	Absorbed by B. suipestifer 107	Absorbed by B. suipestifer 49	Absorbed by B. paratyphosus B 135	Absorbed by Intermediate 201	Absorbed by B. suipestifer 49	Absorbed by Intermediate 91	Absorbed by B. paratyphosus B 135
B. suipestifer Type 49, 11 virus strains, Rabbit 107	1:30000	1:30000	....	1:750	1:15000	....	....	....	....
B. paratyphosus B, Human B96, Rabbit 124, 135	....	....	....	....	....	....	1:15000	1:15000	....
Intermediate, Rabbit 91 Virus 201, 202	....	1:15000	....	1:7500	1:30000	....	....	....	....

The results indicate a close relationship between the intermediate strains and typical B. suipestifer, and to a lesser degree with B. paratyphosus B. So close was the agglutinative affinity between the intermediate strains and B. suipestifer that, with liberal allowance for cultural variation, these strains might be regarded as aberrant strains of B. suipestifer. As noted,[1] the cultural evidence of the identity of strains 134, 149, and 124, 135 with human strain of B. paratyphosus A and B, respectively, is also supported by these agglutination tests. These strains were further studied by absorption of agglutinins (table 4).

It is immediately apparent that the three intermediate strains constitute a distinct type, incapable of absorbing specific agglutinins from B. suipestifer antiserum. Peculiarly, however, both B. suipestifer and B. paratyphosus B can, to a certain extent, absorb agglutinins from an antiserum against the intermediate strains.

SUMMARY

After the fifth day following inoculation with hog cholera virus there was a marked increase in the proportion of cholera infected hogs having gas-producing bacteria in the blood.

Hog cholera blood containing gas-producing bacteria was usually fatal to rabbits when injected subcutaneously.

Hog cholera blood not containing gas-producing bacteria was nearly uniformly harmless to rabbits when injected subcutaneously.

Hog cholera blood containing gas-producing bacteria was no more harmful than blood not containing gas-producing bacteria when injected in large quantities intravenously into hogs in the production of antihog cholera serum.

One strain of B. paratyphosus A was isolated by direct plating of hog cholera blood; another strain of B. paratyphosus A, and two strains of B. paratyphosus B, were isolated from the heart blood of rabbits dying following inoculation with hog cholera blood. These strains were identical in all respect with human strains of B. paratyphosus A and B.

Three identical intermediates were isolated, two by direct plating of hog cholera blood, and one from heart blood of a rabbit which died after inoculation with hog cholera blood. This same type has been isolated before and since from the spleens of pigs.

STUDIES IN EPIDEMIC (LETHARGIC) ENCEPHALITIS

CULTURAL STUDIES

LEO LOEWE AND ISRAEL STRAUSS

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During the course of investigations carried on the past seventeen months, Strauss, Hirshfeld and Loewe,[1] and later Loewe, Hirshfeld and Strauss,[2] demonstrated that epidemic encephalitis was due to a filterable virus. This naturally suggested the application of methods for the cultivation of the filtrate virus. In a brief preliminary note [3] we described a filterable organism obtained from the virus with special cultural methods. On Feb. 11, 1920, before the New York Pathological Society, we presented in detail the experimental work on which this not was based.

In this communication we wish to review this work and to report additional studies carried out since that time.

LITERATURE

The literature concerning the etiology of this disease is contradictory. For the most part, the bacteriologic studies on blood, brain and cerebrospinal fluid have been negative. McIntosh [4] investigated cases of epidemic encephalitis with special reference to *B. botulinus* with entirely negative results. Recently he has succeeded in reproducing encephalitis in a monkey by injecting the Berkefeld filtrate of brain material from a fatal case.

Von Wiesner,[5] in Economo's Clinic, demonstrated a gram-positive diplostreptococcus in smears and cultures with which he claimed to have had positive results in animal inoculations.

Crookshank [6] expressed the belief that von Wiesner's organism was identical with the organism described by Rosenow [7] in 1916. The same organism was isolated from several of the English cases. Von Wiesner's work has received no further confirmation. the prevailing opinion being that he was dealing with a contamination.

Bradford, Bashford, and Wilson [8] report the finding of filtrable organisms in seven diseases, including epidemic encephalitis, trench fever and influenza. The bacteriologic studies were carried out with the aid of a modified Noguchi

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[1] New York Med. Jour., 1919, 109, p. 772.
[2] Jour. Infect. Dis., 1919, 25, p. 378.
[3] Loewe and Strauss: Jour. Am. Med. Assn., 1919, 73, p. 1056.
[4] Brit. Jour. Exper. Path., 1920, 1, p. 2.
[5] Wien. klin. Wchnschr. 1917, 30, p. 933.
[6] Lancet, 1918, 1, p. 653; 1918, 1, p. 699; 1919, 1, p. 79.
[7] Jour. Am. Med. Assn., 1916, 67, p. 1202.
[8] Quart. Jour. Med., Oxford, 1918, 12, p. 99. 9 pl.

tissue medium, using glycerolated brain and brain filtrate as inocula. The work on trench fever and influenza was questioned by Arkwright[9] following a study of Wilson's original cultures and smears. Both Bradford and Wilson in a note appended to Arkwright's article retract their claims as to having recovered in pure culture the agents of these two diseases. Their work on encephalitis is open to the same criticism, especially in view of inconclusive animal inoculations. However, we cannot help feeling that these investigators were proceeding along proper lines.

Brasher and others[10] found an organism in smears of the spinal fluid of two cases which was not the diplostreptococcus of von Wiesner, but a minute gram-positive coccus identical with that isolated by Bradford and others in cases of trench fever, influenza, and trench nephritis. Burnell[11] isolated from the blood in seven cases a gram-negative bacillus which was not motile and not sporeforming, and which he classified as belonging to the hemorrhagic septicemia group, or pasteurelloses. The author states that his findings are inconclusive, but warrant further investigation. Stafford[12] reports finding in smears of centrifugalized sediment of two spinal fluids, from the same patient, rather large, gram-positive diplococci which were cultivated with great difficulty on aerobic mediums only. Animal inoculations were negative.

Morse and Crump[13] report the isolation from the brains of six consecutive cases of lethargic encephalitis, a staphylococcus-like organism, resembling that described by Stafford. The organism was recovered in pure culture from the ventricular fluid. The organism is a gram-positive, non-motile coccus, morphologically like the staphylococcus. It is grown readily on the ordinary laboratory mediums. Their animal experiments were limited, being confined to the subdural inoculation of bouillon cultures into a few rabbits. Encephalitis was apparently produced, but detailed descriptions of the lesions are wanting. They concluded that the encephalitis is produced by a toxin generated by the growth of the organism and present in the filtrate of the cultures. No mention is made of an attempt to grow the filtered cultures on tissue ascitic fluid medium. In view of the readiness with which this organism was recovered, it is rather strange that it has not been found more universally.

Cleland and Campbell,[14] although obtaining negative bacteriologic findings, have stated definitely that in view of their positive animal inoculations, the causative organism is a filter passer with an affinity for the central nervous system. These investigators assert that they were not dealing with epidemic encephalitis, although some of their protocols would suggest that they were dealing with this disease.

Levaditi and Harvier[15] have confirmed the fact that epidemic encephalitis is due to a filtrable virus. They have reproduced our experiments in rabbits and monkeys, and in addition they have found that guinea-pigs are susceptible to this virus. Cultural experiments on the ordinary laboratory mediums were negative. They apparently did not make use of the anaerobic tissue ascitic fluid method.

9 Brit. Med. Jour. 1919, 1, 233.

10 Brasher, Caldwell and Coombe: Brit. Med. Jour., 1919, 1, p. 733.

11 Med. Jour. Austral., 1917, 2, p. 157.

12 Jour. Lab. and Clin. Med. 1919, 4, p. 11.

13 Jour. Lab. and Clin. Med., 1920, 5, p. 5.

14 Med. Jour. Australia, 1919. 1, p. 234.

15 Comptes Rendus des Seances de la Soc. de Biologie, 1920, 83, p. 354; Soc. Med. des Hopitaux, 1920, pp. 179 and 583.

METHODS EMPLOYED

Our early negative cultural studies using ordinary laboratory mediums for aerobic and anaerobic culture, as well as the Rosenow' technic, soon convinced us that we must be dealing with an organism of discriminating cultural requirements. This led us to the adoption of the tissue ascitic fluid medium introduced by Theobald Smith, developed by Noguchi, and so successfully used by him for the cultivation of Treponema pallidum and other organisms, and by Flexner and Noguchi in the growing the globoid bodies of poliomyelitis.

The original Noguchi technic has been followed carefully and is now being used exclusively as the organisms were evidently too highly parasitic to thrive on the modified mediums which suggested themselves.' Plain, salt poor and dextrose broths were used as diluents, but with unsatisfactory results. Human, horse and rabbit serums, and inactivated ascitic fluid were all inimical to the growth of the organism. Due to the apparent resistance of monkeys to this disease it was not deemed wise to attempt the use of monkey serum. Solid and semisolid mediums proved unfavorable for the isolation of organisms directly from infectious material. These modifications were attempted for a two-fold purpose; first, to obtain a more luxuriant growth, and second, to conserve our supply of ascitic fluid. It was also found that the kidney tissue was essential.

The preparation of the medium as now used is briefly as follows:

Sterile kidney fragments are transferred to tubes 20 cm. x 1 1.2 cm., covered with 3 to 4 c.c. of sterile ascitic fluid, and incubated for 48 hours. At the end of this time the contaminated tubes are detected by gross examination and by dark field illumination, and discarded forthwith.

Successful cultivation depends in no small degree on the choice of a suitable ascitic fluid. We feel that we owe no small part of our success to the fact that our ascitic fluid had been stored over a relatively long period of time, and by virtue of its concentration was unusually rich in the elements necessary for the growth of the particular organism with which we were working. The ascitic fluid should conform to certain standards—it should be sterile, bile free, and of a high specific gravity. The presence of fibrin enhances the growth of the organism. Our most luxuriant cultures were obtained with ascitic fluid drawn from decompensated cardiac cases. Abdominal fluid from cases of cirrhosis of the liver can be used, providing

especial care has been taken to determine that traces of bile are not present. A negative blood Wassermann reaction had been obtained in all cases. Ascitic fluid secondary to peritoneal tuberculosis or carcinomatosis does not afford a suitable culture medium. In the final analysis the choice of an optimum ascitic fluid is an empirical one. It has been our custom to use several fluids in cultivating a given material.

There are no special precautions to be observed as regards the kidneys except that it must be removed in a sterile fashion. The kidneys may be extracted by the abdominal route or through lumbar incisions. Occasionally one finds an infected kidney, even after observing the strictest asepsis. Sterile testes from rabbits can also be used. As pointed out by others, the kidney serves a dual purpose—first, to afford special form of nutrient and second, to create a more perfect anaerobiosis.

The tubes containing sterile kidney tissue and ascitic fluid are inoculated, and ascitic fluid added to form a column about 10 cm. high. Petrolatum of low melting point is heated and poured over the surface of the fluid in a layer about 1 cm. thick. The petrolatum quickly cools and effectually seals the tube, producing almost perfect anaerobic conditions. In the beginning the cultures were layered with sterile liquid albolene, which necessitated the use of Novy jars. The use of petrolatum instead of liquid albolene has rendered unnecessary the use of the Novy jars. The petrolatum used in our work is autoclaved in small Erlenmeyer flasks for one hour at 15 lbs pressure. A number of tubes are always inoculated with a given material and usually with different amounts. As the frequent opening of the tubes during the preparation of the medium offers many opportunities for contamination, it is necessary to use the utmost care during the whole process, and it is only by the use of numerous controls that false results can be guarded against. Control tubes are set up as follows: (1) serum and petrolatum, (2) inoculum, serum and petrolatum, (3) serum, kidney and petrolatum.

Both controls and inoculated tubes are incubated at 37 C.

The optimum solid medium is of a gelatinous consistency, made so by the addition of 1 part of 2% nutrient agar to 4 or 5 parts of ascitic fluid, the kidney tissue being added as usual. The customary controls are also made.

The following method of examining and subcultivating solid cultures was suggested to us by Dr. Noguchi: The cotton stopper is first paraffined. A diamond pencil is drawn horizontally across the test tube just below the region to be subcultivated. The tube is immersed, not longer than a minute, in a hot solution containing equal parts of 95% alcohol and saturated bichlorid of mercury. A heated glass rod is applied to the center of the scratch mark made by the diamond pencil. This causes the tube to crack sharply around its entire circumference. The ends of the tube are then separated for a distance of 1 cm. and the agar column gently broken at the desired place. The exposed agar of the upper fragment is dipped in the alcohol-bichlorid solution. The portion of the agar which has become clouded is scraped away with a platinum spatula. By pressing on the paraffined stopper as much agar is extruded as is desired. The tube is then inverted and placed in a shallow receptacle. Single colonies are picked with sterile capillary pipets, if necessary, with the aid of a magnifying glass. The pipets containing the single colonies are washed in tubes of fluid medium, which are then treated in the usual fashion. Smears are made by crushing between two slides bits of agar containing colonies.

MATERIAL CULTURED

The materials cultivated were:

1. Fragments, salt solution emulsions and Berkefeld filtrates of salt solution emulsions of brains of patients and of inoculated animals. It has been our aim whenever possible to use the fragments, since they require little manipulation, and for other reasons to be mentioned. This has been possible in only a small percentage of human brains, and a somewhat larger percentage of brains of animals, due both to the presence of antemortem invaders, and to contamination in the process of removal of the brains. Blocks of brain were taken preferably from the midbrain where the most pronounced lesions of this disease are found. Material for inoculation was removed with the strictest sterile precautions and with the brain in situ. Berkefeld filtrates were used in practically all instances, especially when there was the slightest possibility of contamination. The filtrates were prepared from 5% salt solution emulsions made by grinding in a sterile mortar with sterile sand. Uncontaminated emulsions were also cultivated. When it is desirable to inoculate a large amount of the original filtrate or

emulsion, we have found it of service to concentrate these in vacuo at 36 C. Grossly contaminated material has been stored in the refrigerator in 50% glycerol, and subsequently cultivated en bloc, or ground up and filtered.

2. Cerebrospinal fluid, removed under sterile precautions, from patients and animals, was cultivated in amounts from 0.5 to 1.0 c.c.

3. Blood was drawn under aseptic precautions.

4. Material from the nasopharynx was obtained from living patients by means of nasal irrigations, pharyngeal swabs, or the West tube. At necropsy nasal mucous membrane and nasopharyngeal mucous membrane were removed by means of the curet. In animals, the whole nasopharyngeal mucous membrane was dissected out. Nasal washings were filtered directly, unless the presence of an excessive amount of mucus made it necessary to shake the washings with glass beads. Pharyngeal swabs were washed in several changes of salt, taking care to use small quantities. The slightly turbid fluid so obtained was then filtered. Nasopharyngeal mucous membrane was finely emulsified by grinding in a mortar with sand and salt solution, and then submitted to filtration.

The filters used in the beginning of the work were the standard Berkefeld 5N or W, but lately, due to difficulty in obtaining Berkefeld filters, we have been using the Mandler filters, which we have found satisfactory as regards their ability to hold back the usual test organisms. As tested by the manufacturer, they will retain 8 to 12 lbs. of air pressure without passing an air bubble, after having been immersed in water 12 hours. As tested by us, they hold back B. prodigiosus under the same conditions obtaining in our experimental filtrations. All filtrates before being used were cultivated on ordinary laboratory mediums to insure sterility.

Controls.—Control studies were carried out on nasal washings, nasopharyngeal mucous membrane, blood, and cerebrospinal fluids of patients suffering from, or dead of, diseases other than lethargic encephalitis. Patients from the surgical wards were preferred so as to minimize the possibility of contact with the disease under investigation. The nasopharyngeal mucous membrane and brains of normal rabbits were also studied. Brains from patients dying of various diseases were used as controls. Spinal fluid from healthy monkeys was used to control the cultural findings.

GROSS APPEARANCE OF THE CULTURES

In spite of the numerous precautions used in the preparation of the culture tubes, a small percentage of contaminations invariably appear. These are for the most part due to the numerous manipulations necessary before sealing the tubes. A small number apparently only make their appearance when all three elements—inoculum, kidney and ascitic fluid—are combined. Most contaminations are readily detected grossly, by the dense clouding of the tube, gas formation, evidence of putrefaction, and the rapid disintegration of the kidney. Other contaminations make their appearance on the aerobic subcultures on ordinary laboratory mediums, which are made whenever a tube is exposed. The gross findings are confirmed by dark field illumination and by examination of smears stained by Gram's method. Generally gross contaminations are detected within a few days after the tubes have been set up.

A successful growth is usually manifested on the fifth to the seventh day by clouding of the medium commencing about the kidney tissue, the outline of which becomes hazy and irregular. The clouding extends upward rapidly to within about 1 cm. of the top of the ascitic fluid column. In a few instances in which blood from the kidney has diffused into the body of the culture, it is gradually decolorized in the presence of a positive culture. The organisms are not held in suspension long, but tend to form clumps which settle to the bottom of the tube, leaving the supernatant fluid clear. The degree of clouding is no criterion of the activity or presence of growth, for occasionally one is surprised to find in smears from relatively clear tubes, numerous organisms. The clouding of the medium is due partly to the growth of the organism itself, and partly to protein precipitation by the acid producted by the organisms. This is especially marked in solid cultures on dextrose serum agar, surrounding the individual colonies.

Transfer to solid mediums can be obtained only with the later generations of the organism. In no instance were we able to grow the organism on solid mediums directly from infectious material. The growth in solid mediums assumes different forms, depending on the adaptibility of the given strain to this type of culture medium. When the organisms are numerous, a diffuse clouding of the medium is observed. Individual colonies can only be made out with a magnifying glass. The initial and most intense clouding takes place in the region

of the kidney and extends upward. When the organisms are few in number, minute colonies may appear only in the region of the kidney, or occasionally scattered throughout the medium. The colonies gradually increase in size so as to become easily recognizable with the naked eye.

MICROSCOPIC STUDY OF THE ORGANISM; MORPHOLOGY

Studies of the fluid cultures under dark field illumination reveal the organisms as minute globular refractile forms, occurring singly, in diploform, chains and clumps, the latter form predominating, especially in the older cultures. These bodies show active Brownian motion, but no true motility. When motile forms are found in the dark field, the culture is at once discarded as contaminated.

In stained smears the organisms appear as minute globular bodies which are arranged singly, in diploform, in chains, and clusters. The chain formation is most marked on solid mediums, due to the fact that they are unfavorable for their growth. This observation is analogous to the Pfaundler phenomenon, according to which typhoid bacilli grown in immune serum tend to form chains. The organism has an average diameter of 0.25 mikrons as measured by the ocular mikrometer. Smaller forms are found in young cultures, and larger more deeply staining degenerated forms are seen in the older cultures. The reaction to Gram's stain depends a great deal on the medium used and on the age of the individual culture. Young cultures and those grown on fluid mediums are mostly gram-positive, while the older cultures and those grown on solid mediums tend at times to be gram-negative. In the early work reliance was placed on Loeffler's alkaline methylene blue for want of a satisfactory Giemsa stain. Subsequently several other stains have been tried out which have proved valuable in establishing a definite tinctorial reaction for the organism, which is of basophilic nature.

STAINING METHODS

Loeffler's Alkaline Methylene Blue: Stain from one to two hours; preparations fixed only in methyl or absolute alcohol. The organisms stain a violet hue, which differentiates them from the blue background. The disadvantage with this method is the densely staining background containing tissue particles, which tend to obscure the field. This method has been used for routine rapid work.

Giemsa solution may be used in two ways: the rapid, and the overnight method. It is only lately that we have been able to secure a satisfactory Giemsa stain. The smears are previously fixed in absolute alcohol for ½ to 1 hour, followed by ether for 2 or 3 minutes. The slides are then immersed in dilute Giemsa 1-10 or 15) and permitted to stain overnight. With a more concentrated solution smears can be stained in ½ hour with the aid of heat, care being taken to heat the slides only to steaming. Giemsa smears are more satisfactory for studying the morphology of the organisms.

Unna's Alkaline Methylene Blue: Stain for 1 hour, after fixation in methyl or absolute alcohol for ½ to 1 hour. Differentiation is accomplished by means of a glycerol-ether solution (equal parts of glycerol-ether, 1 part, to 4 parts distilled water). We have obtained a selective stain by this method whereby the organisms stand out as dense purplish bodies on a clear background.

Ljubinsky's Pyoktannin-Acetic Acid: Stain for 45 minutes, after fixation in methyl or absolute alcohol for ½ to 1 hour. Care must be taken in the use of this method since most of the preparation is lost through the solvent action of the acetic acid. The organisms appear dark blue to black.

Unna-Pappenheim: Stain for 1 to 1½ hours, after fixation in absolute alcohol or equal parts of absolute alcohol and saturated mercuric chlorid. This stain serves best for impression smears of brain material. The organisms stand out red against the green of the surrounding tissue.

Pyronin, 1% Aqueous solution: For prolonged staining (overnight). The organisms stain red.

We have occasionally experienced difficulty with precipitate and sediment in these stains. When this occurred the precipitate was removed by centrifugalization, filtration, or, if necessary, by filtration through a Berkefeld candle. We wish to emphasize that heat was never used in the fixation of smears.

RESULTS OF CULTURAL STUDIES

Human Nasopharyngeal Membrane Recovered at Necropsy.—The organism has been recovered in pure culture from Berkefeld filtrates of nasopharyngeal mucous membrane of 7 fatal human cases of epidemic encephalitis. In 10 attempts we have been successful in 7. One

of these strains has been carried to the sixteenth generation and another to the twelfth generation, in artificial cultures, without animal passage. The later strains proved pathogenic for animals.

Controls of filtrates of nasopharyngeal mucous membranes of patients dying of other conditions (cardiovascular disease, megacolon, peritonitis, mediastinal tumor, carcinoma of the stomach, post-operative hemorhhage and empyema) have all been sterile.

Human Nasopharyngeal Washings.—Filtrates of nasopharyngeal washings from 23 cases of epidemic encephalitis were cultivated with

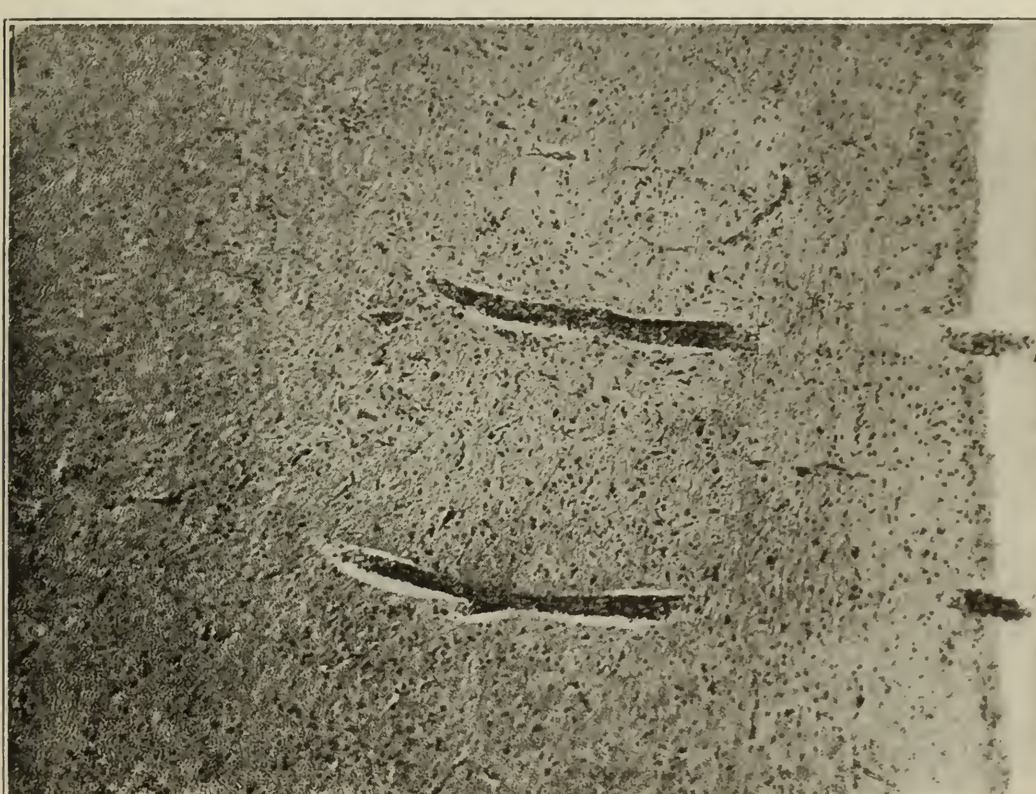

Fig. 1.—Monkey brain, section of pons showing perivascular (adventitial) infiltration with mononuclear cells. Animal injected intracranially with culture originally derived from Berkefeld filtrate of human nasopharyngeal mucous membrane removed at necropsy.

positive findings in 15 cases or 66%. Many of these strains were subcultured successfully and carried along through several generations. The organism was recovered from brains of rabbits injected with the virus of those nasal washings in 8 instances, as well as from the brains of rabbits injected with the organisms from these nasal washings in 5 instances.

Control studies of nasal washings were negative in 8 cases (mastoiditis (2), sinusitis, pyelitis, appendicitis, empyema, cholelithiasis and nephrolithiasis).

Rabbit Nasopharyngeal Mucous Membrane.—The organism was recovered from the nasopharyngeal mucous membrane of 3 rabbits injected with (1) human mucous membrane filtrate, (2) sixth generation of virus from a human mucous membrane, (3) culture from human mucous membrane in sixth generation. Those cultures have been carried, one to the sixth, and the others to the third generation.

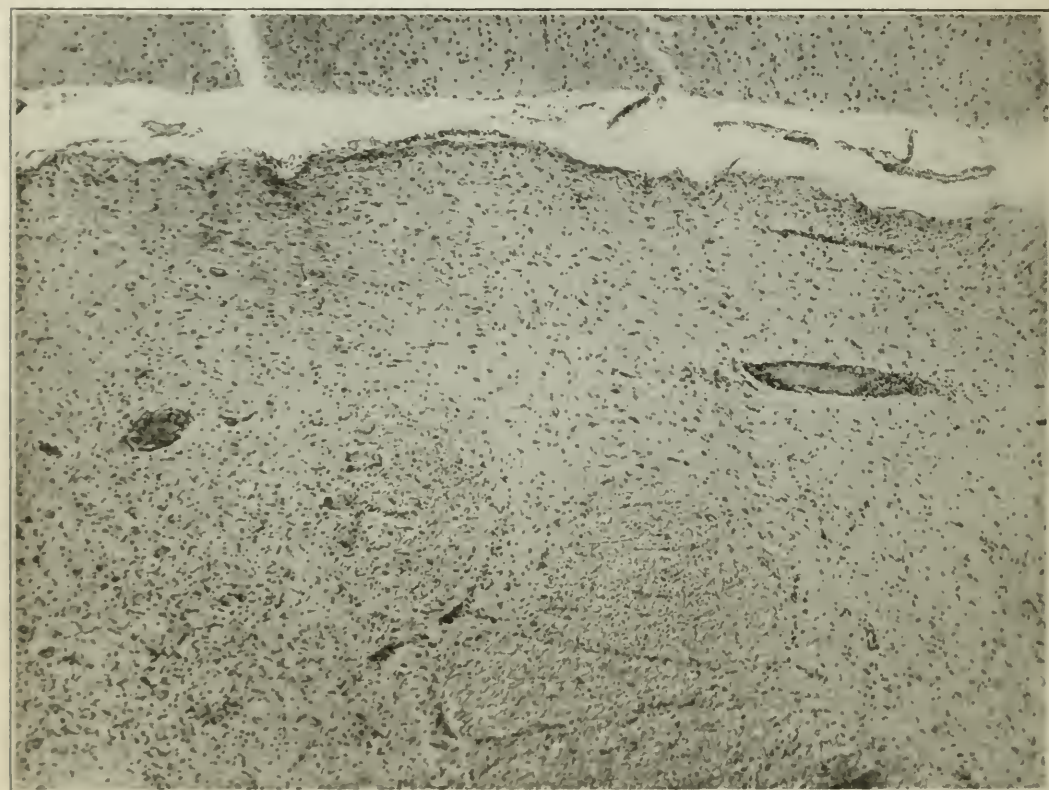

Fig. 2.—Monkey brain; collars of round cells about vessels near floor of fourth ventricle. Animal inoculated intracranially with culture derived from Berkefeld filtrate of human nasopharyngeal mucous membrane removed at necropsy.

The organism was recovered in 2 instances from the brains of animals injected with the Berkefeld filtrate of these rabbit mucous membranes, and in 2 instances from the brains of rabbits injected with the organism derived from these rabbit mucous membranes.

Cultures of Berkefeld filtrate of nasopharyngeal mucous membrane from 7 normal rabbits were used to control these cultures and gave negative results.

Cerebrospinal Fluids.—Cerebrospinal fluids have yielded the organism in 12 out of 24 cases. It was found in one case on direct smear of the sediment of the centrifugalized spinal fluid. These strains were carried in one instance as far as the eighth generation; in another as far as the fourth; but for the most part only through the second generation, as this was deemed sufficient to prove the viability of the culture. The organism was recovered from the brains of 8 rabbits injected with the spinal fluid itself, and from the brains of 4 rabbits injected with the organism derived from these spinal fluids.

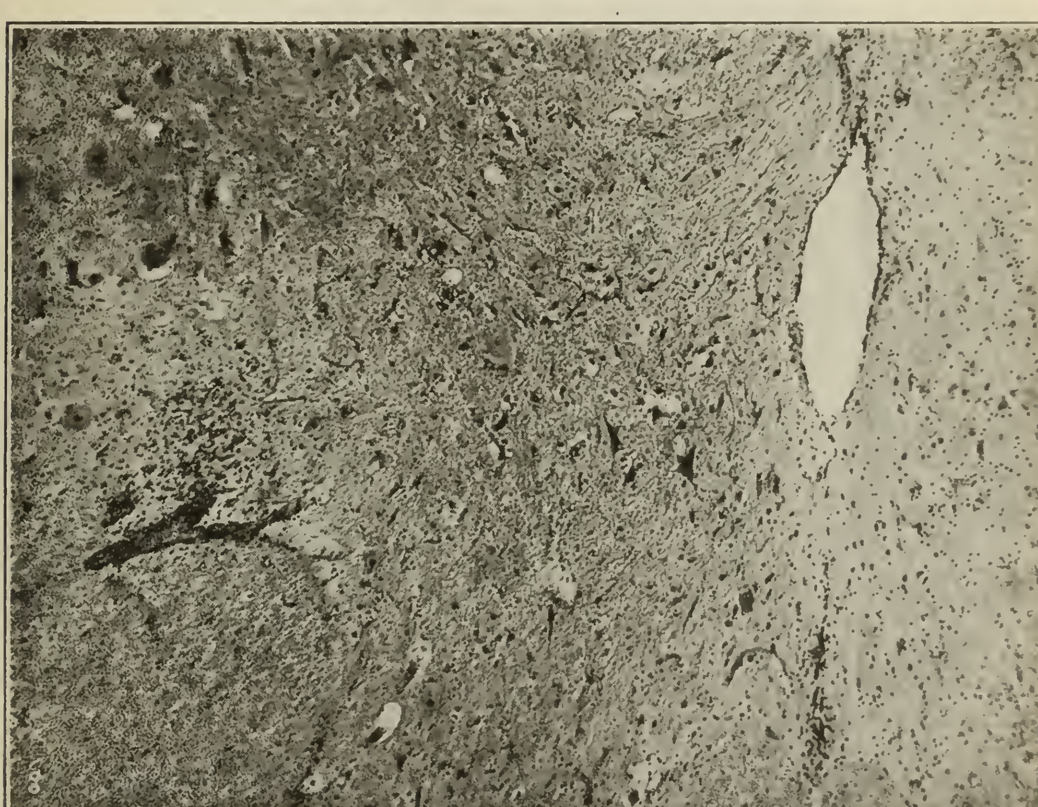

Fig. 3.—Rabbit cord; section of upper dorsal cord. Focus of round cells in proximity to vessel showing adventitial infiltration with mononuclear cells. Animal inoculated intracranially with culture obtained from Berkefeld filtrate of human nasopharyngeal mucous membrane removed at necropsy.

Eight spinal fluids from patients suffering from various diseases than epidemic encephalitis (brain abcess, brain tumor, psychasthenia, uremia, multiple sclerosis, tuberculous meningitis, neurosyphilis, and spinal cord tumor) were cultivated with entirely negative results. Animal inoculations were likewise entirely negative in 6 cases. Cultures and animal inoculations of spinal fluid were made in most cases immediately after withdrawal of the fluid.

Rabbit Brains.—A total of 56 rabbit brains were cultivated, using Berkefeld filtrates of brain, blocks of brains and emulsions of brain; there were 36 positive results, 64%. Ten emulsions were inoculated with 3 positive results. Eleven blocks of brain were inoculated with 10 positive results. The relatively small number of blocks and emulsions cultivated is due to the difficulty in removing the material in sterile fashion. In 3 instances, blocks of brain gave positive results when filtrates did not. This givest a total successful recovery of the organism from 39 of 56 rabbit brains cultured, 69%. There was one

Fig. 4.—Rabbit brain; area showing mononuclear infiltration of adventitia (Virchow-Robins space) of blood vessels in subcortex. Animal inoculated intracranially with culture of cerebrospinal fluid from human case.

series of 7 animal transmission initiated by a Berkefeld filtrate of a human nasopharyngeal mucous membrane. The organism was recovered following each transmission. One of the organisms recovered from this series was injected into animals in the fourth, fifth, sixth, seventh and eighth generations, and was recultivated from 50% of the brains so inoculated. Rabbits were injected with the fourth, fifth, sixth, eighth and eleventh generations of a culture from a human

nasopharyngeal mucous membrane and the organism was recovered from the brains of 4 of these animals, or 50%. A 3 months old glycerolated filtrate from human nasopharyngeal mucous membrane was injected into animals and carried through two transmissions, and the organism recovered from 50% of the rabbit brains. The same

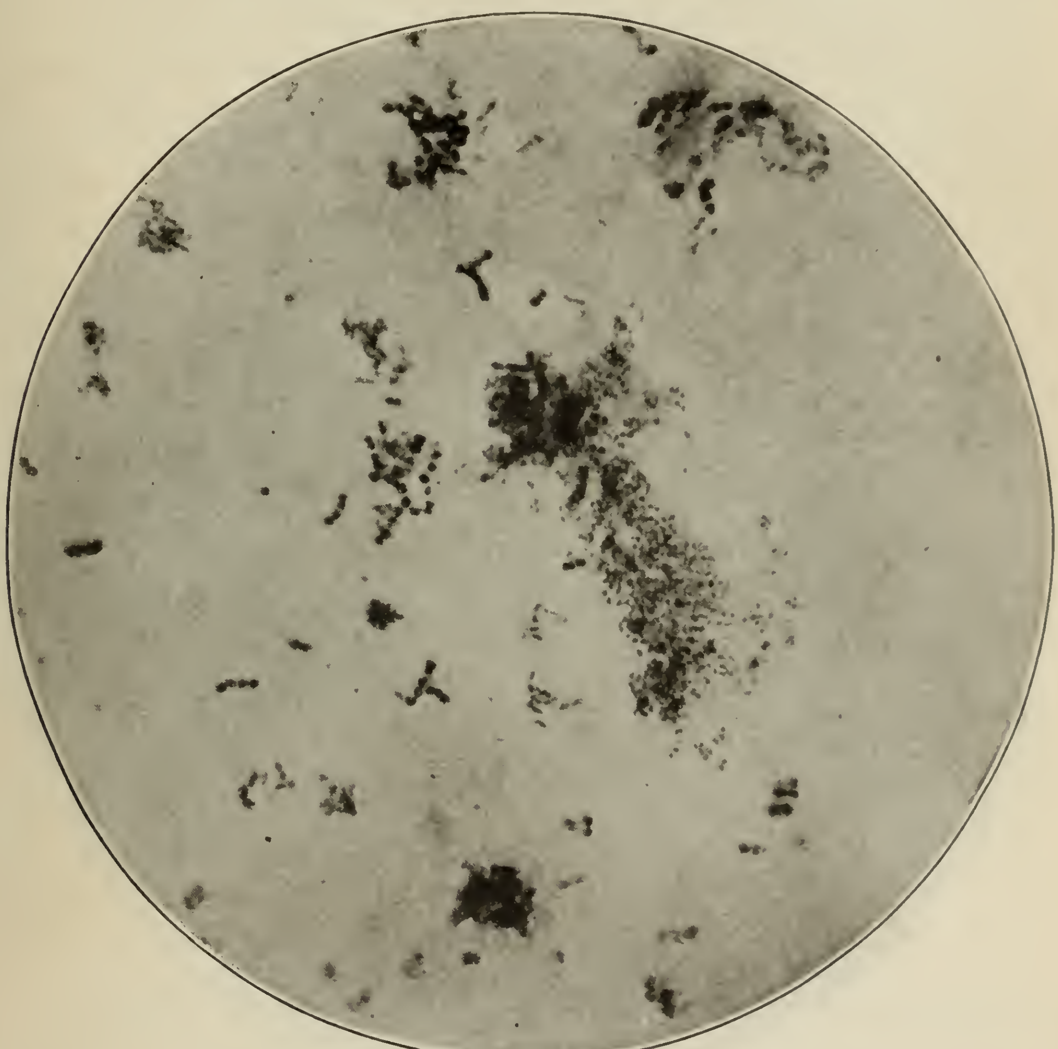

Fig. 5.—Seven day old fluid culture; superimposed anhemolytic streptococcus for comparison; × 1200.

filtrate when 4 months old was injected into 2 animals, with recovery of the organism from both.

In a series of transmissions initiated by spinal fluid from a case of encephalitis, the organism was recovered from the brain in each of the transmissions, the positive results making a total of 5 of the 8

brains cultivated. The second generation of one of the recovered organisms was injected into animals. Cultures of the brains of these animals were positive.

Monkey Brains.—Cultures were made of 6 monkey brains injected with virus of various kinds (filtrates of human nasopharyngeal mucous membrane, and of infected nervous tissue from rabbit, monkey and

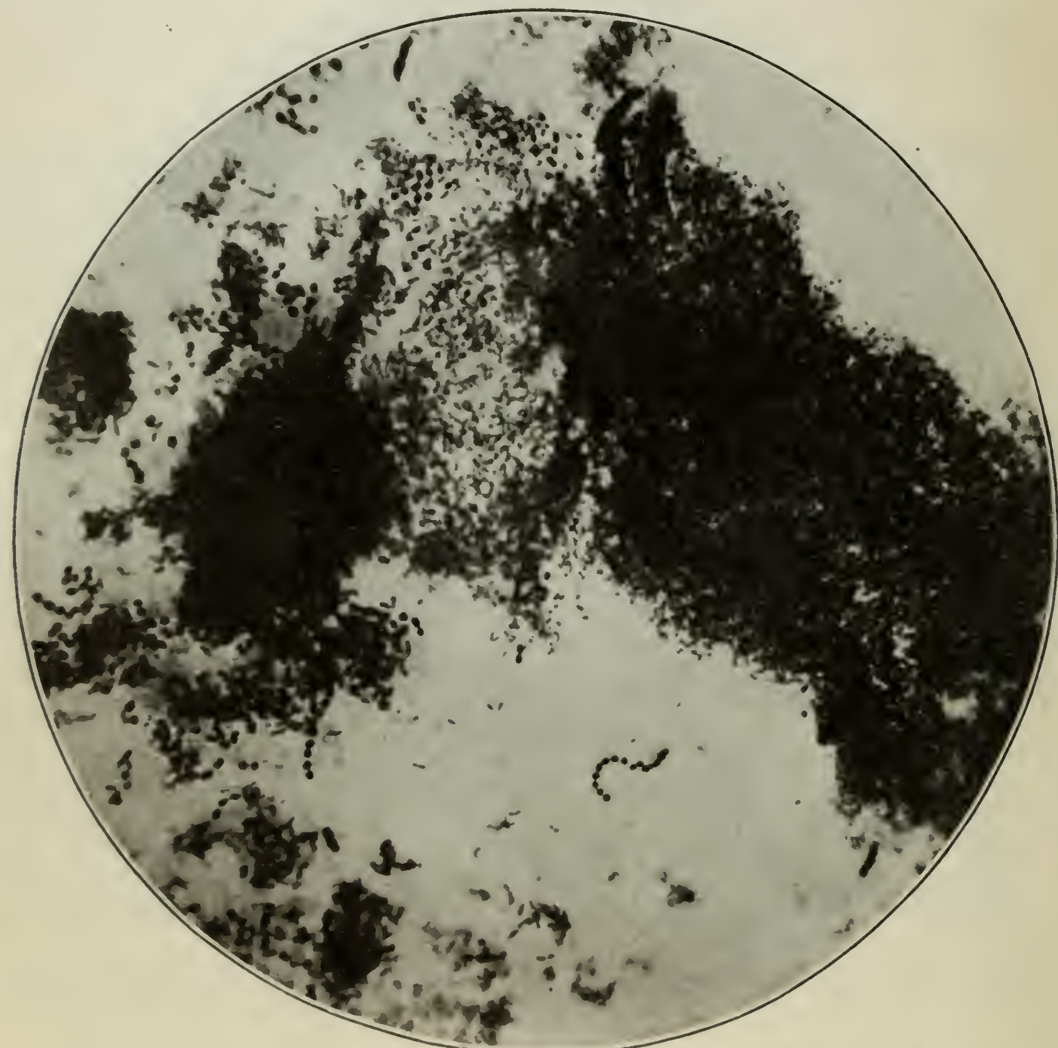

Fig. 6.—Fluid culture 3 weeks old; superimposed anhemolytic streptococcus for comparison; × 1200.

human) and 5 positive results were obtained. The organism was demonstrated in cultures of blocks of a glycerolated brain kept in the refrigerator for 3½ months. It was isolated from the brain of a monkey which developed a secondary B. subtilis infection after a subdural inoculation with the Noguchi culture of a human naso-

pharyngeal mucous membrane. The presence of the B. subtilis was not detrimental to the organism. A positive culture was obtained from the brain of a monkey that was injected with the seventh generation of a Noguchi culture of human nasopharyngeal mucous membrane.

Human Brains.—After numerous attempts and by various means, we have finally been able to isolate the organism from human brains. We achieved our earliest and best results with a case of lethargic encephalitis that ran a rapid course, death ensuing in 5 days. Positive cultures were obtained on the first attempt by the use of large blocks of tissue. Subcultures of this organism proved pathogenic for rabbits. In another case, we were able to use only filtrates because the brain was grossly contaminated. This probably accounts for the great difficulty experienced in isolating the organism. In a third instance, an emulsion of a human brain was successfully cultivated. The original emulsion produced the typical clinical and pathologic pictures in the monkey. Our results have shown that the organism is more readily recovered from the brains of those cases that run a rapid course. Occasionally we have resorted to concentration in vacuo in order to obtain successful growths.

Blood.—Cultural studies of the blood were carried out in two cases and in both the organism was recovered. Both of these cases were of the wild maniacal type with myoclonus. In one case the infection was so overwhelming that death ensued within six days.

The tubes were inoculated at the bedside without the use of anticoagulants. In one case positive cultures were also obtained by the use of a large quantity of blood laked with sterile distilled water. The organisms were separated from fragmented blood cells by repeated subcultures.

PATHOGENICITY OF CULTURES

As in the case of the virus, monkeys are apparently refractory to cultures of the organism. The two following protocols are of interest:

Monkey 17 was injected subdurally with 2 c c of Berkefeld filtrate of the culture of human nasopharyngeal mucous membrane. The culture was of the tenth generation; the individual culture being 3 weeks old. Complete paralysis of both hind legs was first noted 10 weeks after the operation. This gradually spread to involve the right foreleg. The spinal fluid contained 68 cells per cmm. Sections of this brain show what are apparently old, healing lesions in the midbrain. There is considerable proliferation of the adventitia cells of the blood vessels. Monkey 20 was injected intracerebrally with 2 c c of a Berkefeld filtrate of the culture of another human nasopharyngeal mucous membrane.

This culture was originally derived from a 50 per cent. glycerated filtrate kept on ice for 3 months, and had been carried through 7 generations. This monkey was well for 3 weeks, and then developed elevation of temperature and apathy. There was profound lethargy and progressive paralysis of all four extremities. Death ensued within 5 days. The pathologic picture was typical of encephalitis. The organism was recovered from the brain.

We have found that the incubation period is increased by prolonged cultivation on artificial medium and also by filtration of the culture, which results in the removal of a considerable number of the organisms.

Our rabbit inoculation experiments have been carried out with a number of strains preferably in the later generations. About 50% have succumbed with typical lesions. The incubation period varies from 2 to 42 days. In view of this apparent natural immunity a series of rabbits were always inoculated, reliance never being placed on inoculation of a single rabbit.

Three strains, derived from filtrates of nasopharyngeal mucous membrane from lethargic encephalitis cases, have been used. Filtrates of cultures from the third to the eleventh generations were injected intracranially into 20 rabbits. Twelve succumbed with typical lesions. Further successful animal transmissions were made with filtrates of these brains. One animal injected intracranially with 0.2 c c of a filtrate of a 6 weeks old culture in the fourth generation, first showed paralysis of both hind legs 5 weeks after inoculation. The paralysis slowly progressed to involve all four extremities. Typical pathologic lesions were present both in the brain and in the spinal cord. One strain partook of the characters of the virus from which it was isolated, in that it tended to produce hemorrhagic lesions.

Colonies picked from solid cultures of these strains have been grown in fluid medium. These cultures when injected intracranially into rabbits have produced lesions in 5 of 8 animals so inoculated. Cultures derived from brains of inoculated animals proved pathogenic for rabbits in approximately half of the animals injected.

Cultures of organisms from cerebrospinal fluids and of organisms isolated from the brains of rabbits injected have produced lesions in 5 of 12 animals inoculated. The infectivity of cerebrospinal fluid seems to be proportionate to the increase in cells.

We wish to bring out in connection with our animal experiments that we have not only produced typical lesions in rabbits with cultures derived from virus of various kinds, but we have also been able in many instances to recover the organism from the brains of animals

so injected and also to produce again the disease in animals with later generations of these same organisms. We were unable to produce lesions in animals injected in the same manner with control cultures.

DISCUSSION

The experimental evidence that has been presented, would indicate an etiologic relation of the filterable organism that we have isolated, to the disease in question. One possible objection that may be raised is that our successful animal inoculations with cultures have been due to carrying over of sufficient original virus to produce the disease. In our method of making transplants, only 0.2 c c of living cultures are used. It can thus be seen that in later generations the amount of virus carried over from the initial culture through the several subcultures is infinitesimal as compared with the amount of virus required originally for successful animal inoculations. Since the time elapsing between a first and eleventh generation is a matter of months, and in view of the rapidity with which virus deteriorates at incubator temperature, it is evident that original virus cannot be responsible for the potency of our cultures. Further, while the cultures lose in virulence through artificial cultivation over a long period, the incubation period in animals is not greatly prolonged. The symptoms and lesions produced by later generations are just as typical as with earlier generation and with virus. With this point in mind the following experiment was made: Two-tenths c c of a virulent culture was inoculated into a tube of tissue ascitic fluid medium, 15 c c in volume; 0.2 c c of the resulting mixture was then transferred to a similar amount of the same medium and so on for 6 dilutions. One c c taken from the fourth and sixth dilutions were inoculated intracranially into each of 6 rabbits, with entirely negative results. It has already been shown that by using solid cultures, we were able to pick single colonies and to grow the organism in pure culture in this way, and to inoculate successfully animals with the cultures so obtained.

The organism isolated resembles in morphology and cultural characteristics that found by Flexner and Noguchi in poliomyelitis. It differs in virulence, occurrence, and particularly in the ability to infect rabbits. Successful inoculations in the monkey and the rabbit have been obtained with the later generation of our organism. The isolation of organisms from spinal fluid sharply differentiates this disease from poliomyelitis. It is of interest that Foster [15] has isolated a fil-

trable organism from common colds which in morphology and growth resemble the globoid bodies of Flexner and Noguchi, and the organism which we have found in epidemic encephalitis.

It is evident that a new field in bacteriology has been opened up by the investigations of Noguchi. We believe that further investigation will prove that there is a group of filtrable organisms, resembling each other in morphology, but possessing distinctly different pathogenic characteristics. New methods must be introduced for the separation of the organisms of this group.

SUMMARY AND CONCLUSIONS

In our reported investigations we have brought forward this evidence:

Berkefeld filtrates of brain material, nasopharyngeal mucous membrane and nasal washings from cases of epidemic encephalitis have produced in rabbits and monkeys lesions typical of this disease. Spinal fluid and blood have also produced the disease experimentally in these animals. Many of these animals have succumbed with the typical picture of epidemic encephalitis. The virus has been passed through many series of animals. It can be preserved for many months in 50% glycerol.

Cultures made on ordinary mediums and by Rosenow's technic have proved negative.

By means of the ascitic-tissue culture methods perfected by Noguchi, we have been able to cultivate a minute, filtrable organism from cases of epidemic encephalitis: brain, nasopharyngeal mucous membrane, nasopharyngeal washings, spinal fluid and blood.

This same organism has been recovered from the brain and nasopharyngeal mucous membrane of animals that have been inoculated with virus and cultures and which have succumbed to the experimental disease. The cultures thus recovered from these animals have produced the disease when injected into other animals and the organism has again been recovered. Positive animal inoculations have been obtained with the eleventh generation of this organism.

Isolated colonies of the organism grown on solid Noguchi medium have been picked and pure fluid cultures secured. These fluid cultures have also produced encephalitis in animals.

Our results indicate that epidemic encephalitis can be differentiated from epidemic poliomyelitis for these reasons: Rabbits are susceptible

to infectious material from epidemic encephalitis and not from poliomyelitis. Monkeys are very susceptible to poliomyelitis and relatively refractory to material from epidemic encephalitis. Spinal fluid from poliomyelitis is innocuous when injected into rabbits and monkeys, whereas spinal fluid from cases of epidemic encephalitis produces in both of these animals lesions typical of the disease.

Control studies have been uniformly negative with material obtained from human patients suffering from or dead of, conditions other than epidemic encephalitis.

OBSERVATIONS ON CHANGES IN VIRULENCE OF HEMOLYTIC STREPTOCOCCI WITH SPECIAL REFERENCE TO IMMUNE REACTIONS

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In the following pages are recorded the results of observations on changes in virulence of hemolytic streptococci produced by animal passage, growth in artificial culture, and certain other conditions. At the same time the reactions with immune serums of streptococcal strains of varying degrees of virulence were studied and the results thus obtained are stated briefly.

Experiments on Virulence of Streptococci

Careful observations were made on the changes in virulence of a streptococcus as it was grown on blood agar. This strain was isolated from a pleural empyema and in the first culture in ascites broth 0.05 c c of a 24-hour growth killed a mouse within 48 hours. The coccus was not taken up by g. pig leukocytes in the presence of normal serum; formed long chains in broth; was facultatively anaerobic, and of the beta hemolytic type. It was passed through 10 mice in succession, and in order to test the virulence, it was now grown from the heart blood and inoculated as follows: The heart was dropped into a tube with 2 c c of salt solution, the tube shaken, then centrifugated, and 0.2 c c of the supernatant fluid after being diluted several times with salt solution was injected into the peritoneal cavity of a mouse, the same quantity being plated on dextrose agar in order to get some idea of the number of bacteria present. Cultures were made in broth one part and inactivated goat serum two parts and in 24 hours at 37 C., after being diluted in salt solution, 0.2 c c was injected into the peritoneal cavity of a mouse, and the same quantity plated on dextrose agar. The suspension from which the cultures were made were first shaken in a tube with small glass balls in order to break up the chains. Similar experiments were made with cultures directly from the heart on 5% goat blood agar, using the bacteria growing above the water of condensation and suspending them in salt solution. The results, which are given in table 1, show that the virulence is reduced at once in artificial culture. Thus 46 streptococci from the heart blood of a mouse killed by the same organism were sufficient to kill, but when grown in serum broth a larger number of cocci were required to kill and when grown on blood agar a still larger number. Apparently serum broth is a much more favorable medium for the conservation of the original virulence than blood agar.

Apparently something in the culture medium reduced the virulence, and the influence of peptone was studied. Streptococci were grown for 24 hours at 37 C. on goat blood agar with 5% and 15% of peptone and without any peptone, and the virulence tested by peritoneal injections in mice, the number of cocci

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being determined as before by the plate method. Before inoculation the culture medium was made strictly neutral. As shown in table 2, it required 84,000 streptococci cultivated on blood agar without peptone to kill a mouse within 24 hours, whereas 12,400 and 18,000 streptococci grown on 5% and 15% peptone blood agar, respectively, constituted a lethal dose. It may be concluded that peptone is not a chief factor in reducing virulence.

TABLE 1

THE EFFECT OF CULTURE ON THE VIRULENCE OF THE STREPTOCOCCUS

Mice	Source and Number of Streptococci Injected and Results		
	From the Heart of a Mouse	24-Hour Serum Broth Culture	24-Hour Agar Broth Culture
1-3	37,400—died within 17 hours	106,000—died within 17 hours	300,000—died within 17 hours
4-6	37,400—died within 17 hours	106,000—died within 24 hours	300,000—died within 17 hours
7-9	3,740—died within 17 hours	10,600—died within 48 hours	30,000—died within 17 hours
10-12	3,740—died within 20 hours	10,600—died within 72 hours	30,000—died within 17 hours
13-15	280—died within 20 hours	696—died within 72 hours	3,000—died within 24 hours
16-18	280—died within 36 hours	696—died within 72 hours	3,000—died within 24 hours
19-21	46—died within 46 hours	109—survived	300—survived
22-24	46—died within 36 hours	109—survived	300—survived
25-27	46—survived	109—survived	30—survived
27-30	46—survived	109—survived	30—survived

TABLE 2

INFLUENCE OF PEPTONE IN BLOOD AGAR ON VIRULENCE OF STREPTOCOCCI

Mice	Amount of Peptone in Blood Agar, Number of Streptococci Injected and Results		
	No Peptone	5% Peptone	15% Peptone
1-3	84,000—died within 17 hours	124,000—died within 17 hours	180,000—died within 17 hours
4-6	84,000—died within 17 hours	124,000—died within 17 hours	180,000—died within 17 hours
7-9	8,400—survived	12,400—died within 24 hours	18,000—died within 24 hours
10-12	8,400—survived	12,400—died within 24 hours	18,000—died within 24 hours
13-15	840—survived	1,240—survived	1,800—survived
16-18	840—survived	1,240—survived	1,800—survived
19-21	84—survived	124—survived	180—survived
22-24	84—survived	124—survived	180—survived

Next, the influence of the reaction of the medium on the virulence was tested. Turro[1] recommended acid culture medium for streptococci because they live longer and maintain their virulence better on an acid medium. I used agar with 1% peptone, 5% goat blood, and of three different reactions, namely, 0.8% acid to phenolphthalein, 0.5% alkalin, and neutral. Table 3 shows

[1] Centralbl. f. Bakteriol. I, O., 1895, 17, p. 864.

that 1,344 streptococci were killed after growth on the alkaline medium, 2,580 after growth on the neutral medium, and only 510 when grown on the acid medium. These results support the conclusion of Turro.

I then tested the influence of oxygen, the virulence being determined as before, after growth on the surface of 5% goat blood agar and anaerobically in the depths of such blood agar tubes, in each case at 37 C. for 24 hours. It was found that at least 8,500 streptococci grown aerobically were required to kill a mouse within from 36 to 48 hours and 1,100 grown anaerobically.

TABLE 3

Influence of Reaction of Culture Medium on Virulence of Streptococci

Mice	Number of Streptococci, Reaction of Medium, and Results		
	Alkaline	Neutral	Acid
1-2	134,400—died within 24 hours	258,000—died within 17 hours	
3-5	134,400—died within 24 hours	258,000—died within 17 hours	
6-8	13,440—died within 25 hours	25,800—died within 17 hours	51,000—died within 17 hours
8-11	13,440—died within 24 hours	25,800—died within 17 hours	51,000—died within 17 hours
11-13	1,344—died within 24 hours	2,580—died within 36 hours	5,100—died within 17 hours
14-16	1,344—died within 48 hours	2,580—died within 48 hours	5,100—died within 17 hours
17-19	1,344—survived	258—survived	510—died within 36 hours
20-22	1,344—survived	258—survived	510—died within 36 hours
25	survived	survived	51—survived
25-27	....	....	51—survived
28-30	....	....	51—survived

TABLE 4

Influence of Oxygen Tension on Virulence of Streptococci

Mice	Manner of Culture, Number of Streptococci, and Results	
	Aerobic Culture	Anaerobic Culture
1-2	85,000—died within 24 hours	110,000—died within 24 hours
3-4	85,000—died within 24 hours	110,000—died within 24 hours
5-6	8,500—died within 36 hours	11,000—died within 24 hours
7-8	8,500—died within 36 hours	11,000—died within 36 hours
9-10	850—survived	1,100—died within 36 hours
10-12	850—survived	1,100—died within 48 hours
13-14	85—survived	110—survived
15-16	85—survived	110—survived

The streptococci used in the following experiment came from a human abscess and had been cultivated for about a year. It was nonvirulent for mice and rabbits, 0.5 c c of a 24-hour serum broth culture injected into the abdomen of a mouse, and 3 c c injected intravenously in a rabbit being without effect. The coccus was strongly hemolytic on blood agar and formed long chains in broth. Intravenous injections were made into a rabbit and the coccus was grown from the heart blood in one part of broth and one part of inactivated rabbit serum (56 C. for 30 minutes). This process was repeated five times, and after the fifth passage 2 rabbits were injected with 0.2 c c per

kilo of a 24-hour serum broth culture, 2 others with 0.1 c c per kilo of the same culture. The rabbits that received 0.2 per kilo died within 3 days while the rabbits that received only 0.1 c c developed diarrhea and became thin. The virulence of the coccus for mice had now increased so that 0.05 c c of the culture used in the injection of the rabbit killed within 24-48 hours, and the streptococcus was now passed in the heart blood through 18 mice in succession, and the virulence tested. It was found that 0.00001 c c of a 24-hour serum broth culture killed within 48 hours. Six rabbits were now injected; 2 received intravenously 0.01 c c of serum broth culture per kilo, but no special effect was noticed; 2 received 0.05 c c per kilo, one dying within 48 hours, the other within 4 days; in the remaining two 0.1 c c per kilo was injected and one died within 36 hours, the other within 4 days. The coccus was then passed through 24 mice, the virulence was tested again and found to be the same as when it had passed through 18 mice, 0.00001 c c of the serum broth culture killing within 48 hours.

TABLE 5

Virulence Test of Streptococcus After Passages Through Mice

Number of Animal	Quantity of Culture	Results
1	0.001	Died within 17 hours
2	0.001	Died within 17 hours
3	0.0001	Died within 48 hours
4	0.0001	Died within 48 hours
5	0.00001	Died within 48 hours
6	0.00001	Died within 48 hours
7	0.000001	Survived
8	0.000001	Survived
9	....	Survived
10	....	Survived

Streptococci in the heart blood of the dead mice.

It may be concluded from these results that an avirulent hemolytic streptococcus grown artificially for some time acquires increased virulence both for rabbits and mice on being passed through rabbits, and that after such passage further passages in mice increases the virulence especially for mice, but also for rabbits; furthermore, that when a certain maximum virulence is attained by animal passage this virulence is not readily changed by further passage through the same animal.

As stated, the fatal dose for mice of the 24-hour serum broth culture of the streptococcus under consideration was 0.00001 c c after it had passed through 24 mice. For guinea-pigs 2 c c of the serum broth culture was fatal within 48 hours (minimum fatal dose not determined). Cultures were made from the heart blood in serum broth and after being passed through 7 guinea-pigs the lethal dose was determined as shown in table 3. It appears that a streptococcus 0.00001 c c of a broth culture of which kills a mouse, became so reduced in virulence after being passed through guinea-pigs that 0.01 c c was required to kill a mouse. We may say that when the maximum virulence of a streptococcus for the mouse is reached, passage through the guinea-pig increases the virulence for the guinea-pig, but decreases the virulence for the mouse. On the other hand, when the virulence for the mouse is increasing but has not attained the maximum, the passage of the streptococcus through the guinea-pig increases the virulence for both the mouse and the guinea-pig.

It has been suggested that streptococci and other bacteria of the same class form toxins when in contact with the tissues of the infected body (Lindemann, Friedberger, Neufeld and Dold, and others), but no definite information in regard to this point is at hand. I have studied the effect on streptococci in collodion sacs placed in the abdominal cavity of rabbits. Caliero[2] concluded that the virulence of streptococci was increased by sojourn in collodion sacs inserted in the abdominal cavity of guinea-pigs, but decreased for rabbits. Tuneoka[3] found that staphylococcus grew in virulence while in sacs in the cavity of rabbits previously immunized with the organism.

The collodion sacs were made in the usual way, care being taken to obtain a thin membrane so that the body fluids surely would pass through. Serum broth (1-1) cultures of streptococci, 24 hours old, were centrifugated and a loop of the sediment suspended in 1 c c of salt solution and a certain quantity introduced into each sac, one being placed in each side of the peritoneal cavity of a rabbit. The streptococcus was the one used in the previous experiment; it was not virulent for mice, rabbits or guinea-pigs, 0.5-1 c c of rabbit serum broth culture could be injected into the peritoneal cavity of a mouse without any effect.

Exper. 1.—One collodion sac with 1 c c and one with 0.5 c c of streptococcus suspension, prepared as described were left in the peritoneal cavity of a rabbit for 6 days and then were opened. One c c of salt solution was introduced into each sac and after mixing it carefully with the white contents, 1 c c was put into each of 2 freshly made sacs, which were then placed in the cavity of a rabbit. A rapid loss of weight followed and after 3 days the rabbit died from peritonitis, not due to streptococci. The sacs were intact, rabbit serum broth cultures were made of the contents, and the virulence tested for mice after 20 hours at 37 C. The lethal dose was found to be 0.3 c c, showing that the virulence had increased during the sojourn in the abdomen of the rabbit.

As I had found previously that the virulence of a streptococcus brought up to the maximum for a particular animal is reduced by passage through another animal, while during the period of increasing virulence passage through another animal may serve still further to increase the virulence for the first animal, I tried to find out whether such changes occur when streptococci are kept in a collodion sac in the abdominal cavity. For this purpose I used the streptococcus obtained from a pleural empyema and cultivated it artificially for about 6 months when it was passed through 10 mice. At the time of the experiment 0.001 c c of a 24-hour rabbit serum broth (1-1) culture was a fatal dose for mice.

Exper 2.—A 24-hour rabbit serum broth culture was centrifugated and the sediment suspended in salt solution, one loopful to 1 c c; of this suspension 1 c c was placed in each sac and 2 sacs were introduced into the abdominal cavity, but the animal died from streptococcus peritonitis, one of the sacs having broken. The intact sac was opened, 1 c c of salt solution added to the contents and 0.5 c c of this suspension were placed in each of 2 sacs which were then introduced into the abdomen of a fresh rabbit where they remained for 2 weeks. When removed there was a small amount of whitish yellow material in the sacs, which were intact, and preparation showed that many of the streptococci remained unstained. Cultures were made in rabbit serum broth and the virulence tested for mice. Before being introduced into the abdomen, the lethal dose was 0.001 c c of a 24-hour serum broth culture; after having been kept in the abdomen for 16 days, the fatal dose was the same. Apparently no change in virulence for mice had taken place.

EXPERIMENTS ON AGGLUTINABILITY OF STREPTOCOCCUS

The question whether the agglutinability of a streptococcus strain changes under different conditions is an important one from the point of view of the grouping of streptococci. As pointed out previously,[4] the use of cinnobar obviates the action of minor agglutinin, at least to some extent, and I have found further that cinnobar also prevents spontaneous agglutination of streptococci. Twenty-four hour cultures of streptococci in 0.2% dextrose broth were centrifugated, the bacteria washed with salt solution, and a suspension made to

[2] Centralbl. f. Bakteriol. I, O, 1914, 47, p. 208.
[3] Nippon Biseibutsu Gakkan Zasshi, 1916, 3, p. 382.
[4] Nakayama, Jour. Infect. Dis., 1919, 24, p. 489.

which a small quantity of cinnobar was added. The tubes were then shaken thoroughly and left to stand for 2 or 3 hours when they were centrifugated for a little while until a homogenous suspension was produced. This treatment not only breaks up the chains, but seems to make the cocci less sensitive to agglutinin. The agglutinating serum was prepared by injecting rabbits with streptococci: twenty-four-hour dextrose broth cultures were centrifugated, the sediment suspended in salt solution and after being heated at 60 C. for one hour, injected intravenously. Three or more injections were then given of the same material, but without being heated, at intervals of 5-7 days, and serum obtained 7-10 days after the last injection.

I now studied the changes in agglutinability of a streptococcus on passage through rabbits. A typical Strep. pyogenes was used. The results are shown in table 6. The serum of the rabbit immunized with the original streptococcus was agglutinating for the original strain in dilution of 400 but for the streptococcus after 3 and 5 rabbit passages in dilution of 50. The serum 1:400 agglutinated the original culture, but after the rabbit passages agglutination was obtained with a dilution of 1:50 only. On the other hand, the serum of a rabbit injected with the streptococcus after it had been passed through 5 rabbits agglutinated this strain in a dilution of 640, the streptococcus in the original culture and after one rabbit passage in a dilution of 320. It would appear that the streptococcus underwent some change on passage through rabbits.

TABLE 6

Agglutination by Various Immune Serums of Streptococcus Passed Through Rabbits

Immune Serum	Streptococci							
	Original Strain		After 1 Rabbit Passage		After 3 Rabbit Passages		After 5 Rabbit Passages	
	2 Hours	20 Hours	2 Hours	20 Hours	2 Hours	20 Hours	2 Hours	20 Hours
Serum of rabbit injected with original culture....	1:400	1:1600	1:200	1:1600	1:50	1:400	1:50	1:400
Serum of rabbit injected with streptococci after 5 rabbit passages.......	1:160	1:320	1:80	1:320	1:80	1:640	1:80	1:640

Figures give highest active dilution of serum.

Next, the agglutination of streptococci after passage through rabbits, mice and guinea-pigs was studied with the results illustrated by table 7. We see that the streptococci were agglutinated more strongly by the strictly homologous serum. It is notable that the serum of a rabbit injected with streptococci after 5 rabbit passages had less agglutinating power with respect to the streptococci treated in other ways, especially those that had passed through guinea-pigs. The serum produced with a streptococcus that had been passed through 5 rabbits and then through 15 mice agglutinated the homologous streptococci, the streptococci in the original state and the streptococci that had been passed through guinea-pigs, but it had little effect on the streptococci treated in other ways. The serum of rabbits injected with streptococci passed through guinea-pigs agglutinated these cocci more strongly than the cocci that had been passed

through 5 rabbits and 24 mice. The results suggest that the streptococci in the original culture were more like the passage streptococci than these resembled each other.

The normal serum of the guinea-pig, rabbit, goat and horse agglutinated all the various streptococci in low dilution.

TABLE 7

CHANGES IN AGGLUTINABILITY OF STREPTOCOCCUS ON ANIMAL PASSAGE

Immune Serum	Streptococci: Original Strain, 2 Hours	Original Strain, 20 Hours	After 5 Rabbit Passages, 2 Hours	After 5 Rabbit Passages, 20 Hours	After 5 Rabbit and 15 Mouse Passages, 2 Hours	After 5 Rabbit and 15 Mouse Passages, 20 Hours	After 5 Rabbit and 24 Mouse Passages, 2 Hours	After 5 Rabbit and 24 Mouse Passages, 20 Hours	After 5 Rabbit, 24 Mouse and 7 Guinea-Pig Passages, 2 Hours	After 5 Rabbit, 24 Mouse and 7 Guinea-Pig Passages, 20 Hours
Serum produced with original strain	1:400	1:1600	1:50	1:400	1:50	1:100	1:50	1:100	1:50	1:100
Serum produced with strain passed through 5 rabbits	1:200	1:800±	1:200	1:1600	1:50	1:200	1:50	1:200	1:50±	1:100±
Serum produced with strain passed through 5 rabbits and 15 mice	1:200	1:800	1:50	1:100	1:400	1:1600	1:50	1:100	1:50	1:100
Serum produced with strain passed through 5 rabbits and 24 mice	1:200	1:800	1:100	1:200	1:50	1:200	1:800	1:1600	1:100	1:800
Serum produced with strain passed through 5 rabbits, 24 mice and 7 guinea-pigs	1:50	1:100	0	1:50	1:50	1:100	1:50	1:200	1:100	1:800

Figures give highest active dilution of serum.

Absorption experiments were made in the following manner: the serum was diluted 10 times with salt solution and 10 loopfuls of the centrifugate of streptococcus cultures in 0.2% dextrose broth added. The suspension was incubated for 2 hours and then centrifugated thoroughly. It was found that when a serum produced by injections of the original streptococcus culture was treated in this manner with this streptococcus strain, all streptococcus agglutinins were removed. After treatment of this serum with streptococci after 5 rabbit and 15 mouse passages, the serum still agglutinated the original streptococcus in a dilution of 1:200, but had no effect on the other streptococcal strains. When the serum was treated with streptococci that had been passed through 5 rabbits, 24 mice and 7 guinea-pigs, it still agglutinated the original streptococci in a dilution of 1:200, and the streptococci that had passed through 5 rabbits in a dilution of 1:100. Furthermore, the treatment of the immune serum produced with streptococci that had passed through 5 rabbits, 24 mice and 7 guinea-pigs with the same streptococcus removed all streptococcus agglutinins, but after treatment with the original streptococcus the serum still agglutinated the strictly homologous streptococcus in a dilution of 1:100 but had no effect on any other streptococci. It may be concluded that the agglutinin are absorbed freely by the streptococci used in the immunization and that the agglutinins that remain vary more or less with respect to their action on related streptococcal strains.

Opsonification of Streptococci

Table 8 gives the results of phagocytosis experiments with the same serum and streptococcal strains as in the agglutination experiments. Guinea-pig leukocytes were used and the mixtures were incubated for 30 minutes. The results show that the opsonins were increased in all the immune serums and that the virulent strains were more resistant than the less virulent, but there is no indication of any fundamental difference between the different strains.

TABLE 8

Opsonification of Streptococci by Immune Serums

Immune Serum	Streptococcus in Original Culture	Streptococcus after Rabbit Passages	Streptococcus after Rabbit and 15 Mouse Passages	Streptococcus after Rabbit and 24 Mouse Passages	Streptococcus after Rabbit, 24 Mouse and Guinea-Pig Passages
Serum 1, immune serum produced with original streptococcus	28.4	17.3	8.0	4.0	3.1
Serum 2, immune serum produced with streptococcus after passage through rabbits	29.6	19.0	8.3	4.5	4.5
Serum 3, immune serum produced with passage through mice (24)	27.9	18.3	10.6	9.7	6.2
Serum 4, immune serum produced with streptococcus after passage through rabbits, mice and guinea-pigs	23.1	17.0	12.8	11.1	8.4
Normal rabbit serum	12.0	10.0	0	0	0

Precipitation Tests

Marmorek[5] showed that specific immune serum may cause precipitate in filtrates of streptococcus cultures. Aronson[6] obtained precipitates with extracts of streptococci in 1% aethylendiamin solution and specific serum, but not with culture filtrate. Eisler[7] obtained precipitates with the concentrated, filtrated and immune serum. Recently Barnes[8] studied the relation between the hemolytic, fermentative and precipitinogenic properties of streptococci, and he concludes that results of the precipitin reaction agree with the results of the hemolytic and fermentative reactions in classifying streptococci. He found, however, that in low dilutions the immune serums would give group reactions with the fluids of streptococcus cultures.

The method I followed was to centrifugate 24-hour cultures in 0.2% dextrose broth, wash the centrifugate with distilled water and then suspend one loopful in 1 c c of water, heat to 60 for one hour, and place in the ice chest for 24 hours. The suspension was then shaken (130 revolutions a minute) for 15 hours and filtered through a maasen filter. Being unable to get definite results with the immune serum used in the agglutination tests, I injected rabbits with the material prepared as just described, beginning with quantities of 1 c c and increasing up to 10 c c, giving the injections intravenously and subcutaneously at intervals of 4-7 days and bleeding 10-12 days after the last injection. In making the test 0.1 c c of immune serum was added to progressive dilutions of the antigen, the total quantity being always made up to 1.1 c c by means of salt solution; the mixtures were then incubated and the result noted at the end of 5, 8 and 24 hours.

[5] Kolle and Wassermann's Handbuch, 1914, 2, p. 787.

[6] Berl. klin. Wchnschr., 1902, p. 979.

[7] Kolle and Wassermann's Handbuch, 1914, 2, p. 784.

[8] Jour. Infect. Dis., 1919, 25, p. 47.

It was found that immune serum was strongly precipitative for the streptococci used in producing the serum and reacted weakly with all the other strains. The nature of the precipitinogen in streptococci apparently changes under different conditions.

Acid Agglutination of Streptococci

According to Michaelis[9] acid agglutination depends on the precipitability of proteins by hydrogen ions. In order to study this form of agglutination of streptococci the following solutions were prepared, each containing normal NaOH solution 5 c c and increasing quantities of normal HCl solution as shown below, the total quantity in each case being made 100 c c by the addition of distilled water:

[9] Deutsche med. Wchnschr., 1911, 37, p. 969.

Solutions	Normal HCl Solution	Quantity of H-ion
1	7.5	1.10-5
2	10	2.5-5
3	15	4.10-5
4	25	8.10-5
5	45	16.10-5
6	85	30.10-5

The centrifugated sediment of 24-hour cultures in 0.2% glucose broth were washed thoroughly with distilled water and finally suspended in distilled water to which a small amount of cinnobar was added. After agitation and standing for a few hours the suspensions were centrifugated a short time in order to remove the cinnobar. In each case 1 c c of streptococcus suspension was mixed with acid solution and incubated for one and a half hours. As shown in table 9, there was little difference in the agglutination of the various strains of streptococci treated in this way.

TABLE 9

Acid Agglutination of Streptococci

Streptococcal Strains	Acid Solutions						
	1	2	3	4	5	6	Distilled Water
Original streptococcus	++	+	+	+	+	±	0
After rabbit passages	+	++	+	+	+	0	0
After rabbit and mice passages	+	++	+	±	0	0	0
After rabbit, mice and guinea-pig passages	+	+	++	+	+	0	0
After 100 mice passages	+	++	+	0	0	0	0
Staphylococcus aureus	+	+	+	++	+	0	0
Pneumococcus	0	0	0	0	0	0	0

Conglutination of Streptococci

Bordet and Gay[10] found that beef serum, heated to 56 C., would cause clumping and increased lysis of blood corpuscles in the presence of inactivated hemolytic serum and complement. Later Bordet and Streng[11] called the sub-

[10] Ann. de l'Inst. Pasteur, 1906, 20, p. 467.

[11] Centralbl. f. Bakteriol., I. O., 1909, 47, p. 260.

stance in the beef serum causing this action conglutinin and Streng found that it acts not only on corpuscles but also on bacteria; furthermore, that it is active in the presence of specific antiserum from which agglutinins have been removed by absorption. The method has been used with some success to differentiate between certain closely related bacteria, but Swift and Thro[12] found that in the case of streptococci conglutination was not of any greater value than ordinary agglutination.

In my experiments I used the same streptococcal suspensions and immune serum (inactivated) as in the agglutination tests. Guinea-pig serum was used as complement, 0.5 c c of a 10% dilution in salt solution were used in each test. Fresh beef serum, after being heated to 56 C. for 30 minutes, was added to each mixture to the amount of 0.5 c c of a 10% dilution in salt solution. The mixtures were incubated for 2 hours. All the reactions were strongest as a rule in the serum mixtures containing the homologous streptococcus.

TABLE 10

CONGLUTINATION AND AGGLUTINATION OF STREPTOCOCCI BY IMMUNE SERUMS

Streptococcal Strains	Immune Serums			
	Serum 1	Serum 2	Serum 3	Serum 4
1. Original streptococcus:				
Conglutination	0 0005	0.001	0.001	0.005
Agglutination	0.0025	0.005	0.005	0.025
2. Streptococcus after rabbit passages:				
Conglutination	0.0005	0.0005	0.025	0.025
Agglutination	0.0025	0.001	0.025	0.025
3. Streptococcus after rabbit and mouse passages:				
Conglutination	0.005	0.01	0.0005	0.01
Agglutination	0.005	0.01	0 0025	0.01
4. Streptococcus after rabbit, mouse (24) and guinea-pig passages:				
Conglutination	0.005	0.025	0.005	0.001
Agglutination	0.001	0.025	0.005	0 0025
Streptococcus 0:				
Conglutination	0.025	0.025	0.025	0.025
Agglutination	0.025	0.025	0.025	0.025
Staphylococcus:				
Conglutination	0	0	0	0
Agglutination	0	0	0	0
Pneumococcus:				
Conglutination	0	0	0	0
Agglutination	0	0	0	0

The figures give the smallest amount of serum causing conglutination and agglutination.

COMPLEMENT FIXATION TESTS

The various strains of streptococci obtained after passages through different animals were used in complement fixation tests in order to determine, if possible, whether there was any difference in their antigen properties. Antisheep rabbit serum was used, the titer being 0.0005 and double this dose was used in the tests. The antigen consisted of the washed centrifugate of 24-hour streptococcus cultures in 0.2% dextrose broth, suspended in distilled water, then heated at 55 C. for one hour and thoroughly shaken in a machine, many small glass balls first being added. The suspension was then centrifugated and the supernatant fluid mixed with 10% salt solution, making a suspension in 0.9% salt solution. The dose of the antigen in the fixation test was 0.5 c c. The same antistreptococcus serum was used as in the agglutinin experiments. The results revealed that no difference between the different streptococcus cultures could be made out.

[12] Arch. Int. Med., 1911, 7, p. 24.

SUMMARY

The virulence of a streptococus rapidly falls on artificial cultivation, particularly on blood agar. The amount of peptone in the medium does not seem to influence the virulence so much as the reaction, acid reaction maintaining virulence better than alkaline. The virulence persists longer in anaerobic than in aerobic conditions.

A streptococcus that has been cultivated artificially for some time and has become avirulent increases in virulence for both rabbits and mice on passage through the rabbit. If also passed through mice, the virulence is further increased, especially for mice, and when a certain maximum in virulence has been reached no further increase develops on further passages through mice.

When maximum virulence for mice has been established, passage through rabbits may increase the virulence for rabbits but decrease it for mice. On the other hand, if virulence for mice is still on the increase, passage through the rabbit may increase the virulence for both rabbits and mice.

Virulence may be increased by keeeping streptococci in a collodion sac in the peritoneal cavity of rabbits.

The agglutinability of a streptococcus may change as the result of animal passage, the particular strain used for immunization being agglutinated more strongly than the related strains by the corresponding immune serum. The original nonvirulent mother streptococcus was agglutinated by all the immune serums. The same relation seems to obtain with reference to opsonins and phagocytosis, as well as with respect to specific precipitation, and conglutination, but no differences could be made out between the different strains by means of complement fixation.

All the various strains were agglutinated in the same way by acid solution.

THE RELATION BETWEEN THE NUMBER OF BACTERIA AND ACID PRODUCTION IN THE FERMENTATION OF XYLOSE *

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The study of the rate of growth of bacteria and the extent of the changes in the composition of the nutrient medium which accompany growth has engaged the attention of bacteriologists for many years. The most important contributions to this problem may be divided along two lines: first, the relation between the number of bacteria and the amount of the end products at different times; second, involved mathematical equations and formulae to explain the rate of multiplication of bacteria in nutrient solutions. The usual method of investigation is to seed a small number of bacteria into a suitable medium under known conditions, and after given intervals of time to estimate the number by the plate method. The rate of development can be obtained for many kinds of bacteria by measuring their products, i. e., the extent of fermentation is assumed to be proportional to the number of bacteria that have developed.

This article is primarily concerned with the rate at which by-products are formed and their relation to the number of bacteria. In order to study the mechanism of fermentation reactions, the rate of development of the micro-organism is of importance. At present, observations are not available which cover the whole period of growth from commencement of seeding to the period when growth ceases altogether, and by-products are no longer formed. It is true that there are ample observations available on the reproduction of bacteria and yeasts in the early stages of growth.

Nägeli and Schwendener [1] were perhaps the first to study the rate of growth of bacteria. They attempted to measure the reproduction of bacteria by the amount of products formed. For this purpose known dilutions of an impure

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[1] Das Mikroskop, 1877, p. 645.

culture of bacteria were prepared and the different dilutions used to inoculate a definite amount of culture medium. They found that the time required to produce a certain amount of by-products, e. g., acid from sugar, is proportional to the bacterial content.

Buchner, Longard, and Riedlin[2] used the Koch plate method in a study of the rate of growth of the cholera vibrio. From the results of counts made at the beginning and at the end of these tests, they calculated the time of generation. Their results indicate that cholera vibrio in a peptone sugar broth culture medium at 37 C., varies in its generation time from 19 to 40 minutes.

Mach and Portelle[3] in a study of alcoholic fermentation by yeast attempted to measure the growth of micro-organisms and the accumulation of their products. Their observations extended over the first 8 days of fermentation. During the first 2 or 3 days they obtained a rapid increase in the number of yeasts present, accompanied by a small production of alcohol and after 3 days the weight of the yeast remained constant, while the amount of alcohol rose rapidly. They determined the amount of yeast present by drying it at 100 C. and then weighing.

Rahn[4] carried out a number of experiments in the study of the influence of by-products on the reproduction of *B. flourescens*. He found that the substance which retards growth in a broth culture is removed when the culture is passed through a porcelain filter, treated with ether, or heated. His results show that this toxic substance is labile. In 1911 Rahn[5] determined the amount of acid formed in one hour by a single cell of Bact. lactis acidi. In the given time a single cell produced a quantity of acid approximately equivalent to its own weight. Rahn maintains that fermentation and growth are parallel in the life of an organism and that the apparent absence of fermentation in young cultures is misleading. The products are present, but in such small amounts that they cannot be measured.

Marshall and Farrand[6] in a study of the bacteria associated in the souring of milk, found that multiplication of bacteria and acid production are not always coincident. They concluded that certain types of organisms commonly found in milk accelerated the growth of the lactic acid bacteria and that other types retarded the production of lactic acid.

Berghaus[7] studied the relation between number of bacteria and ammonia production in a 1% peptone broth medium. Cultures of B. proteus, B. coli, B. typhosus, B. fecalis alcaligenes, B. prodigiosus, and cholera vibrio were used. He found that the bacteria multiply enormously within the first day or two, then decrease slowly. In general, the maximum number is found within 24 hours after inoculation. The formation of ammonia was not parallel to cell reproduction but reached a maximum many days after the bacteria showed a marked decline in number. Berghaus concluded that ammonia formation was not dependent on reproductive cells but might be produced by living, nonreproductive cells or by the substances given off from the dead cell.

Barber[8] studied the rate of reproduction of B. coli at different temperatures. By means of a capillary pipet he isolated a single organism and deter-

[2] Centralbl. Bakteriol., I, 1887, 2, p. 1.
[3] Landw. Vers-Station, 1892, 41, p. 261.
[4] Centralbl. Bacteriol., II, 1906, 16, pp. 417 and 609.
[5] Mich. Agric. Exper. Station Tech. Bull. 10, 1911.
[6] Centralbl. Bakteriol., II, 1908, 21, p. 7.
[7] Archiv. Hyg., 1908, 44, p. 3
[8] J. Infect. Dis., 1908, 5, p. 379.

mined its rate of multiplication by direct observation under the microscope. He found no lag period in the growth of the organism when it was placed in a medium to which it was accustomed.

Lane-Claypon,[9] Penfold,[10] Ledingham and Penfold,[11] and Slator,[12] studied the mechanics of reproduction of micro-organisms in nutrient solutions and under the influence of certain factors. The mathematical equations governing the different phases of growth were discussed in detail.

According to Buchanan,[13] there are seven relatively distinct phases or periods in the life of a bacterial culture. Different equations are given for these seven phases in the development of a culture. Buchanan presents in this paper a review of the literature and a discussion of the mathematical equations.

A recent publication by Baker and his collaborators[14] compares the rate of bacterial multiplication with the rate of acid production in milk. They determined the number of bacteria present both by the plate method and by the direct microscopic count. Since lactic acid is one of the principal products formed by certain organisms in the souring of milk and in the fermentation of xylose, the data obtained by Baker and his co-workers are in some respects comparable to those which will be presented in this paper.

The experiments described were made in an attempt to determine the relations between the number of bacteria present and the rate of acid production during the fermentation of xylose. Experiments were planned to estimate the rate of growth of bacteria by means of plate counts and direct counts, and by the measurements of total acidity. The micro-organism chosen for this experiment was Lactobacillus pentoaceticus, an acetic and lactic acid producer, which occurs on plant tissue, silage, and manure. This micro-organism possesses many desirable points. It grows rapidly in a suitable medium, forms distinct, small, nonspreading colonies, is easily stained, and attacks the pentose sugar, xylose, rapidly, forming almost equal quantities of acetic and lactic acids. Some of the characters of this organism have been described in previous papers from this station.[15] Observations were also made to determine whether there was any change in the ratio between volatile and nonvolatile acids with increase in the

[9] J. Hyg., 1909, 9, p. 239.

[10] J. Hyg., 1914, 14, p. 215.

[11] J. Hyg., 1914, 14, p. 242.

[12] Biochem. J., 1913, 7, p. 197; Trans. Chem. Soc., 1916, 109, p. 2; J. Hyg., 1917, 16 p. 100; J. Soc. Chem. Ind., 1919, 38, p 391.

[13] J. Infect. Dis., 1918, 23, p. 109.

[14] N. Y. Agric. Exper. Station, Tech. Bull 74, 1919.

[15] Fred, E. B.; Peterson, W. H., and Davenport, Audrey, J. Biol. Chem., 1919, 39, p. 347; 1920, 41, p. 431; 42, p. 273.

age of the culture. Comparisons were made of the plate method of counting bacteria with the direct method of Breed.[16]

A 2% solution of xylose in a water extract of compressed yeast was prepared and sterilized for 30 minutes at 15 pounds' pressure. The culture medium was inoculated with pure culture of Lactobacillus pentoaceticus and incubated at 28 C.

TABLE 1

Relation Between Number of Bacteria and Acid Production from Xylose in the Absence of Calcium Carbonate

Number	Hours Since Inoculation	Bacteria in 1 c c of Culture, Plate Counts	Total Titrable Acidity, 0.1 N in 100 c c of Culture, C c	Titrable Acidity by Bacteria, 0.1 N in 100 c c of Culture, C c
1	0	11,000,000	7.8	0.0
2	4	....	7.8	0.0
3	8	80,000,000	8.8	1.0
4	12	....	12.6	4.8
5	16	....	17.8	10.0
6	18	203,000,000	20.9	13.1
7	20	220,000,000	22.0	14.2
8	22	....	24.3	16.5
9	24	273,000,000	24.9	17.1
10	26	....	26.9	19.1
11	28	....	29.2	21.4
12	30	....	29.8	22.0
13	34	277,000,000	36.3	28.5
14	42	290,000,000	38.4	30.6
15	48	440,000,000	42.4	34.6
16	54	....	44.7	36.9
17	58	....	45.1	37.3
18	70	320,000,000	50.3	42.5
19	82	165,000,000	54.0	46.2
20	96	90,000,000	61.5	53.7
21	190	....	94.5	86.7

In the first experiment, the number of bacteria present was determined by the plate method, and in the second experiment both by the plate method and by the direct method of Breed. Because of the nature of the culture it was found necessary to employ a fixative for the direct microscopic preparation. Such a fixative was prepared by mixing equal portions of egg albumin and glycerol, and filtering through paper pulp. A very thin film of this solution was first dried on the slide, after which 0.01 c c of the culture solution was spread over 1 sq. cm. and then dried. With methylene blue the background stained a light blue and organisms a much darker blue. No attempt was made to count each individual cell in the microscopic preparations, if it occurred in a conglomerate, since groups of several organisms would give rise to a single colony on an agar plate. The figures obtained in the second experiment showed agreement between the two methods.

[16] N. Y. Agric. Exper. Station, Tech. Bull. 49, 1916.

The volatile acids were determined by distilling the culture with steam and titrating the distillate with 0.1 N barium hydroxid in the presence of phenolphthalein. The distillation was continued until less than 0.5 c c of barium hydroxid was required for the titration of 100 c c of distillate. After the removal of the volatile acids, the residue was evaporated to about 100 c c on a steam bath, and the nonvolatile acid extracted with ether by means of a Kutscher and Steudel extraction apparatus. This extraction was continued for about 72 hours. The flask containing the ether extract was then disconnected,

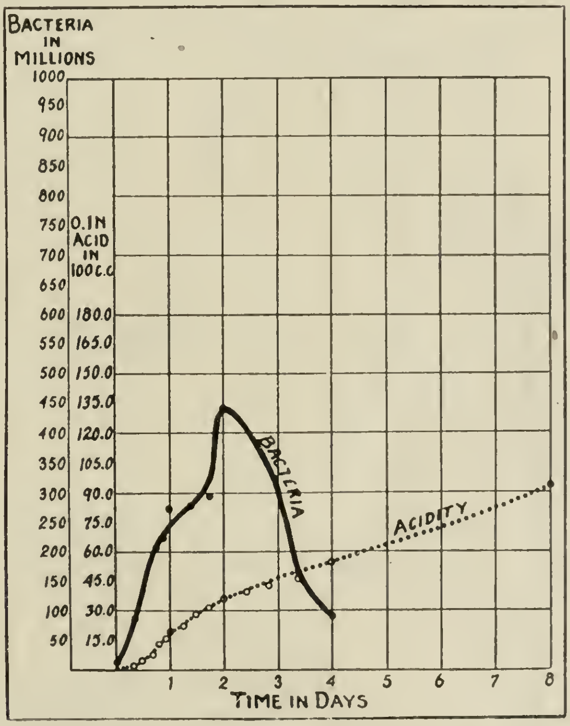

Chart 1.—Curves showing relation between total acidity and number of bacteria in the culture.

20 or 30 c c of water added and ether carefully distilled off. The solution was titrated while hot with 0.1 N barium hydroxid. Fred, Peterson and Davenport [15] found that the volatile acid obtained from the fermentation of xylose by Lactobacillus pentoaceticus is acetic, and the nonvolatile acid is lactic. Therefore, the only attempt made to identify the acids obtained was in the second experiment, in which the Duclaux constants of the volatile acid obtained from a 69-day old culture were compared with those obtained by Fred, Peterson, and Davenport from a 21-day old culture.

Experiment 1.—In this experiment 800 c c of a 2% xylose yeast water solution was inoculated with 10 c c of a 24-hour old liquid culture of Lactobacillus pentoaceticus, and incubated at 28 C. At the time of inoculation and at varying intervals thereafter, the number of bacteria per c c was determined. For this purpose, 1 c c of the solution was removed with a sterile pipet, proper dilutions made, and plates poured. In a similar manner 10 c c portions were removed at

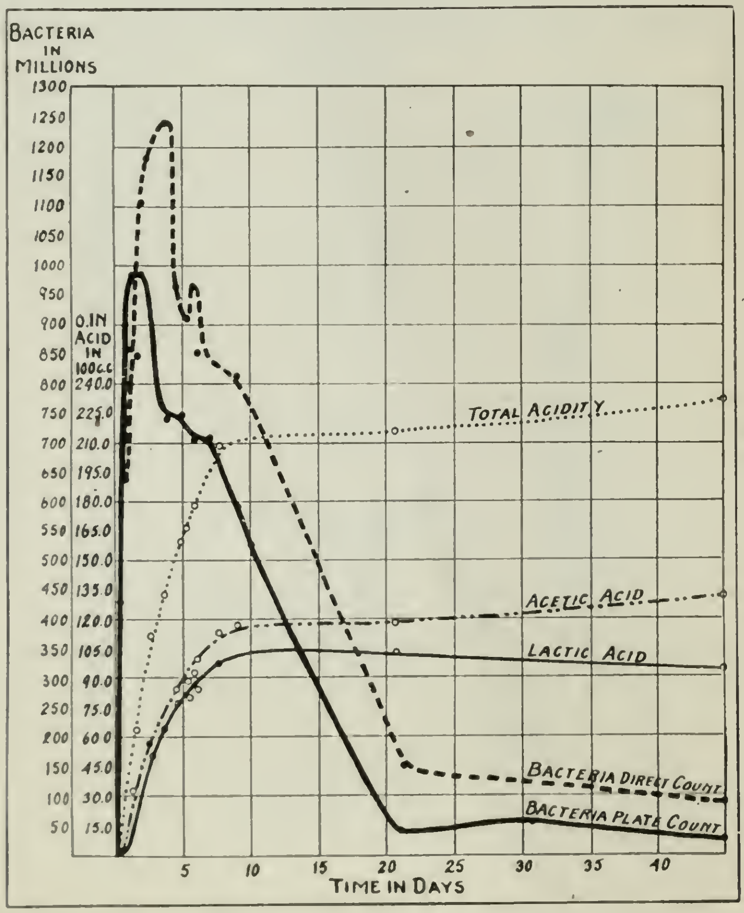

Chart 2.—Curves showing relation between number of bacteria and acidity.

frequent intervals and titrated with 0.1 N barium hydroxid. These data are shown in table 1. According to these results, the period of most rapid multiplication occurred during the first 18 hours after inoculation, when the number increased from 11,000,000 to 203,000,000. From the eighteenth to the forty-second hour the bacteria multiplied rather slowly, and then made a rapid increase between the forty-second and forty-eighth hours, when the maximum number of 440,000,000

organisms was reached. After this time the count gradually decreased until at the end of 96 hours only 90,000,000 bacteria were present.

The acidity rose most rapidly during that period in which the most rapid multiplication of bacteria took place. This is perhaps more clearly shown by chart I than by table 1. When the acidity reached a concentration of about 0.05 N the bacteria decreased in number very rapidly while the acidity increased slowly until, after 190 hours, the culture was very nearly a 0.1 N solution of acid.

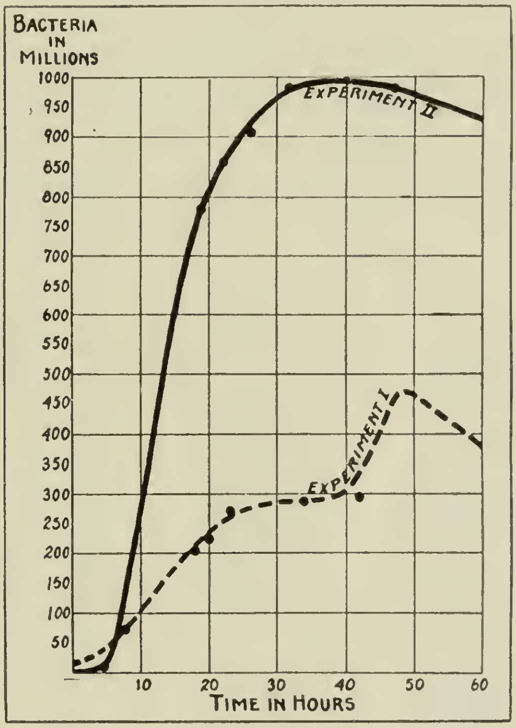

Chart 3.—Curves showing effect of calcium carbonate on number of bacteria. Exp. I. no $CaCO_3$ Exp. II with $CaCO_3$.

Experiment 2.—Three liters of a 2% xylose yeast-water solution were prepared in the same manner as in the previous experiment, except that after sterilization, an excess of calcium carbonate was added to the solution. It was then inoculated with 25 c c of a 24-hour culture of Lactobacillus pentoaceticus. Samples were removed from time to time by means of a sterile pipet for plate counts, and for the determination of volatile and nonvolatile acids. The results are shown numerically in table 2 and graphically in chart 2.

In this experiment a comparison was made between the plate method and Breed's direct method of counting bacteria. The direct count

was usually made from a dilution of 1 : 100. When 1 : 100 c c of such a dilution was spread over 1 sq. cm. the microscopic fields could easily be counted. The fair agreement between the plate and direct counts is due to the fact that no attempt was made to count individual organisms. The higher counts obtained by the direct method are undoubtedly due to more than one factor, e. g., cells may stain which do not have the power of reproduction in agar. The direct count and the plate count show the same general increase and decrease. In the case of the plate count the maximum is reached at the end

TABLE 2

Relation Between Number of Bacteria and Acid Production from Xylose in the Presence of Calcium Carbonate

No.	Hours Since Inoculation	Bacteria in 1 c c of Culture		0.1 N Acid in 100 c c of Culture		
		Plate Count	Direct Count	Volatile, C c	Nonvolatile, C c	Total, C c
1	0	580,000	....	0.0	0.0	0.0
2	5	7,600,000	34,500,000			
3	15	....	430,000,000	0.59	0.30	0.89
4	19	775,000,000	639,000,000			
5	23	857,000,000				
6	27	907,000,000	....	11.15		
7	30	967,000,000	800,000,000			
8	39	....	842,000,000	32.93	30.70	63.63
9	43	850,000,000	929,000,000			
10	48	967,000,000	1,109,000,000			
11	64	....	1,188,000,000	57.25	52.30	109.55
12	88	877,000,000	1,245,000,000	69.40	63.40	132.80
13	91	740,000,000	1,099,000,000			
14	111	....	962,000,000	83.70	76.10	169.80
15	124	747,000,000	909,000,000	88.20	77.40	165.60
16	136	....	968,000,000	91.60	85.40	177.00
17	148	703,000,000	849,000,000	102.00		
18	166	707,000,000				
19	187	....	....	113.00	96.15	209.15
20	216	593,000,000	816,000,000	115.80		
21	244	533,000,000				
22	520	45,000,000	153,000,000	114.50	100.95	215.45
23	740	58,000,000	....	122.30		
24	1079	27,000,000	....	133.00	95.50	228.50
25	1652	12,500,000	56,600,000	146.90	93.10	240.00

of about 30 hours and continues at this high level for about 18 hours when a gradual decline occurs. By the direct count the maximum number is reached somewhat later but attains a higher point. The presence of a neutralizing agent, calcium carbonate, permits an increased multiplication of the bacteria and a more nearly complete fermentation. If the rate of acid production in the second experiment be compared with that of the first, it apparently rises much more slowly. Perhaps this difference is more apparent than real. In the second experiment, calcium carbonate was present which prevented a direct titration of the acid. The method used in this second experi-

ment no doubt does not measure accurately small amounts of acid. Moreover, in the presence of this basic substance, the longevity of the organisms is greater. An analysis for sugar was made on the culture at the conclusion of the experiment 69 days after inoculation, but only a trace of sugar was found. Fred, Peterson, and Davenport obtained a fermentation of about 80 to 90% of the sugar in the presence of calcium carbonate. The completeness of the fermentation in exper. 2 is perhaps due largely to the age of the culture. As in the previous experiment, the acidity increased rapidly for a short time after the number of bacteria had reached the maximum. A comparison between the increase in number of bacteria found in expers. 1 and 2 is shown by the curves of chart 3. The curve obtained in exper. 1 shows how strikingly the addition of calcium carbonate affects the increase in the number of bacteria in a xylose yeast-water culture medium.

TABLE 3

DISTILLING CONSTANTS OF THE VOLATILE ACIDS OBTAINED BY THE DUCLAUX METHOD

	Age of Culture, Days	10 C c	20 C c	30 C c	40 C c	50 C c	60 C c	70 C c	80 C c	90 C c	100 C c
Culture 118-8	21	7.5	15.6	23.7	32.2	41.3	50.8	61.1	72.2	84.7	100
Culture 118-8	69	7.7	15.9	24.4	33.2	42.3	51.9	62.2	73.3	85.7	100
Duclaux constant for acetic acid..	..	7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100
Difference.....	21	+0.1	+0.4	+0.3	+0.2	+0.4	+0.3	+0.5	+0.3	+0.3	
Difference.....	69	+0.3	+0.7	+1.0	+1.2	+1.4	+1.4	+1.6	+1.4	+1.3	

In the curves for lactic and acetic acid, chart 2, it will be seen that after 21 days the amount of acetic acid increased and the lactic acid decreased slightly. The last analyses for lactic and acetic acids are not indicated in the chart but are shown in table 2. This change in the ratio of acetic to lactic acid is due to a secondary fermentation which began after practically all of the xylose had been fermented. A similar destruction of lactic acid in old cultures of Lactobacillus pentoaceticus was noted by Peterson and Fred in the fermentation of glucose, fructose and mannitol. They have also demonstrated the ability of this organism to ferment solutions of sodium lactate with the formation of acetic acid and carbon dioxid. The disappearance

of lactic acid from old cultures of other organisms was previously noted by Kayser,[17] Gayon and Dubourg,[18] and Duchacek.[19]

Nature of the Volatile Acid.—Because of the age of the culture, 69 days, it was thought that possibly volatile acids other than acetic might be formed. In order to test this point Duclaux analyses were made of the volatile acid residues. These constants compared well with those obtained by Fred, Peterson, and Davenport [15] (1919) in a 21-day old culture, as is shown in table 3. The results indicated that acetic acid was undoubtedly the only volatile acid produced during fermentation. The difference between the Duclaux constants obtained after 69 days and those obtained after 21 days was perhaps due to the difference in the original yeast-water, which usually contains a very small amount of volatile acid, the identity of which is unknown.

DISCUSSION

The results of these investigations and those obtained by others show that the fermenting capacity of a culture is determined by the number and age of the bacteria present. At first the rate of fermentation parallels the multiplication of the organisms. Perhaps this is due to the fact that the fermenting capacity of young cells is greater than that of old cells. There are fewer bacteria in an old culture and from a comparison of the rate of acid formed in a given time it appears that the cells have a diminished fermenting capacity. These organisms may form compounds other than the main products of fermentation, which are toxic to their growth.

Slator [12] found that the low oxygen tension brought about by the carbon dioxid produced by yeasts was injurious to the cells before the concentration of alcohol became high enough to be harmful. Perhaps a somewhat similar condition is found in the case of Lactobacillus pentoaceticus but with a substance other than CO_2.

Rubner [20] says that growth of bacteria is comparable to that noted in animals; namely, that reproduction is a characteristic of young cells which may be followed by a long period of active existence in which the cell continues to function. Growth and life after growth are but stages in the general functions of a cell. As shown by the

[17] Ann. de l'Inst., Pasteur, 1894, 8, p. 737.
[18] Ann. de l'Inst. Pasteur, 1894, 8, p. 108; 1901, 15, p. 527.
[19] Centralbl. Bakteriol., I, 1904, 37, p. 326.
[20] Archiv. Hyg., 1906, 57, p. 161.

yeasts, many micro-organisms possess the power of bringing about metabolic changes without reproduction. It must be remembered that by means of enzymes given off from the cell, bacteria can bring about changes that result in a preparation of the food for the cell. Thus fermentation may be concerned with autolytic processes, brought about by the cells that are unable to reproduce. That the temperature at which optimum reproduction occurs is not necessarily the optimum temperature for the production of byproducts has been clearly demonstrated by a number of investigators. The most favorable temperature for fermentation by Lactobacillus pentoaceticus is 28 C. Whether this temperature is also the most favorable for reproduction has not been determined.

It has been stated by others that fermentation does not occur until some time after the inoculation of a culture and that the length of time before fermentation begins is dependent on the temperature, age, and activity of the cells, adaptability of the organism to its new environment, and size of the inoculum. The fact that so few organisms are present as to make a detection of their fermentation products impossible, does not necessarily mean that no fermentation has taken place. Since fermentation may be coincident with growth and reproduction, or may occur without reproduction, it seems safe to conclude that fermentation may commence as soon as a solution is inoculated with an organism.

CONCLUSIONS

The rate of acid production in the fermentation of xylose by Lactobacillus pentoaceticus is most rapid during the period of maximum growth of the bacteria. A decline in the rate of growth is accompanied by a decrease in acid production although a slow acid formation is noted for many days.

In the first stages of growth, the curves of multiplication of bacteria and of acid formation are almost parallel although growth precedes the formation of acid in measurable quantities. The maximum number of bacteria occurs in the early stages of fermentation, usually within 48 hours after inoculation, while the maximum acidity is not noted for several days. In a xylose yeast-water medium the bacteria multiply more rapidly and reach a higher number if calcium carbonate is present.

In old xylose cultures of Lactobacillus pentoaceticus, the ratio between lactic and acetic acids may change. This is probably due to a secondary fermentation of the lactic to acetic acid.

Apparently, acetic acid is the only volatile acid produced in the fermentation of xylose by Lactobacillus pentoaceticus.

Direct count and plate count of the total number of bacteria give the same general results.

A LEPROSY-LIKE DISEASE IN THE LUNGS OF A MEXICAN PARROT

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A parrot, called by the dealers "Mexican Red Head," died a month after inoculation with scarlet fever blood. It appeared to be normal throughout except that the lungs contained many scattered grayish tubercle-like nodules about 0.5 to 1 mm. in diameter. Smears stained by the tubercle method showed numerous acid-fast bacilli which resembled the tubercle or lepra bacillus. Many of these, however, were short or even coccoid.

Sections showed that these tubercles were largely peribronchial (Fig. 1). They were composed of epithelioid cells with vesicular nuclei (Fig. 2). Some cells contained two nuclei. In the sections stained with hematoxylin and erythrosin the new growths appeared to be purely epithelial of epithelioid proliferations without any changes in the surrounding tissues and without leukocytic infiltration; nor did they show any connective tissue stroma or capsule. However, the surrounding tissues were greatly congested and in places there was extensive hemorrhage.

In sections stained with carbol fuchsin, decolorized with 2% hydrochloric acid in 95% alcohol, and then stained with Unna's polychrome methylene blue, the epithelioid cells were seen to be filled with acid-fast bacilli lying at all angles. Now the picture was very much like that of human or rat leprosy (Figs. 3 and 4).

Many, but not all, of the growths contained so many black, amorphous particles, like carbon, that the cellular structure and the bacilli could be seen with difficulty. Some of the unpigmented, as well as the pigmented tumors were near a bronchus.

Before the tissue was cut it was thought that the lesions were those of avian tuberculosis. Cultures on rabbit-blood agar and glycerol potato were kept under aerobic, partial tension and anaerobic conditions at 37 C. and 24 C. for six months. There was no growth.

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Fig. 1.—Leprosy-like disease in a parrot.

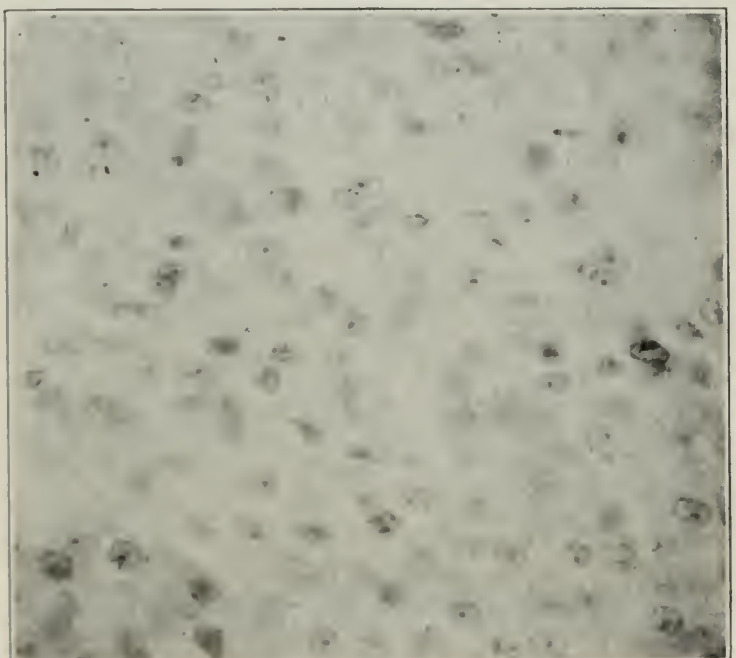

Fig. 2.—Leprosy like disease in a parrot.

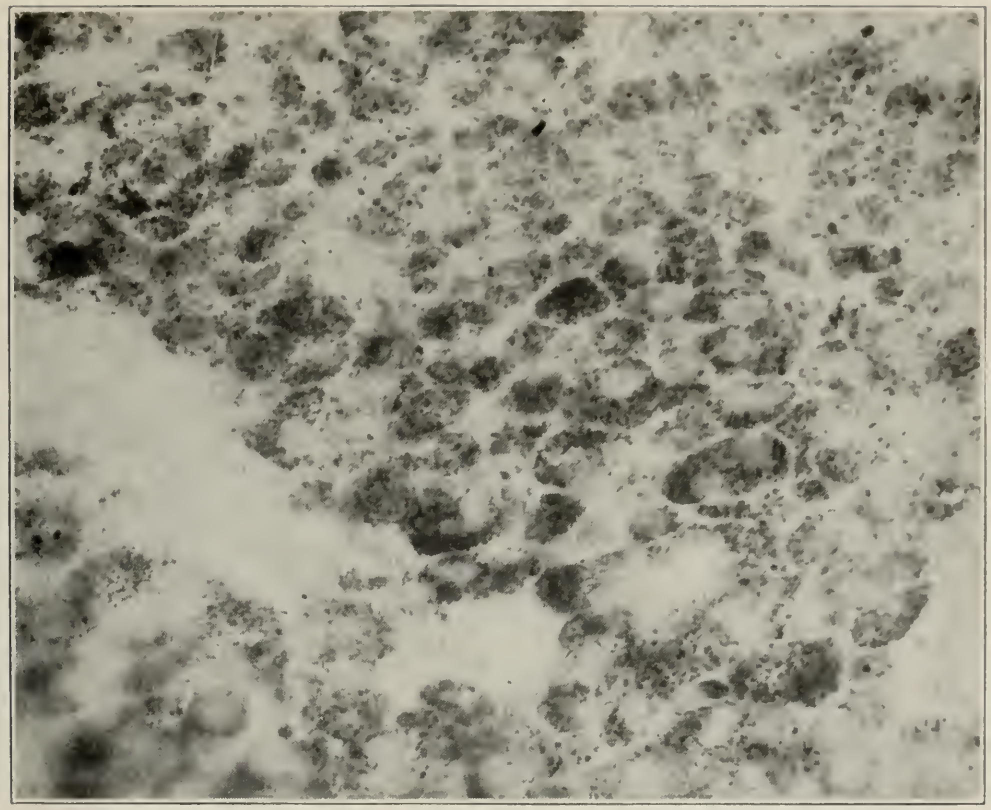

Fig. 3.—Leprosy-like disease in a parrot.

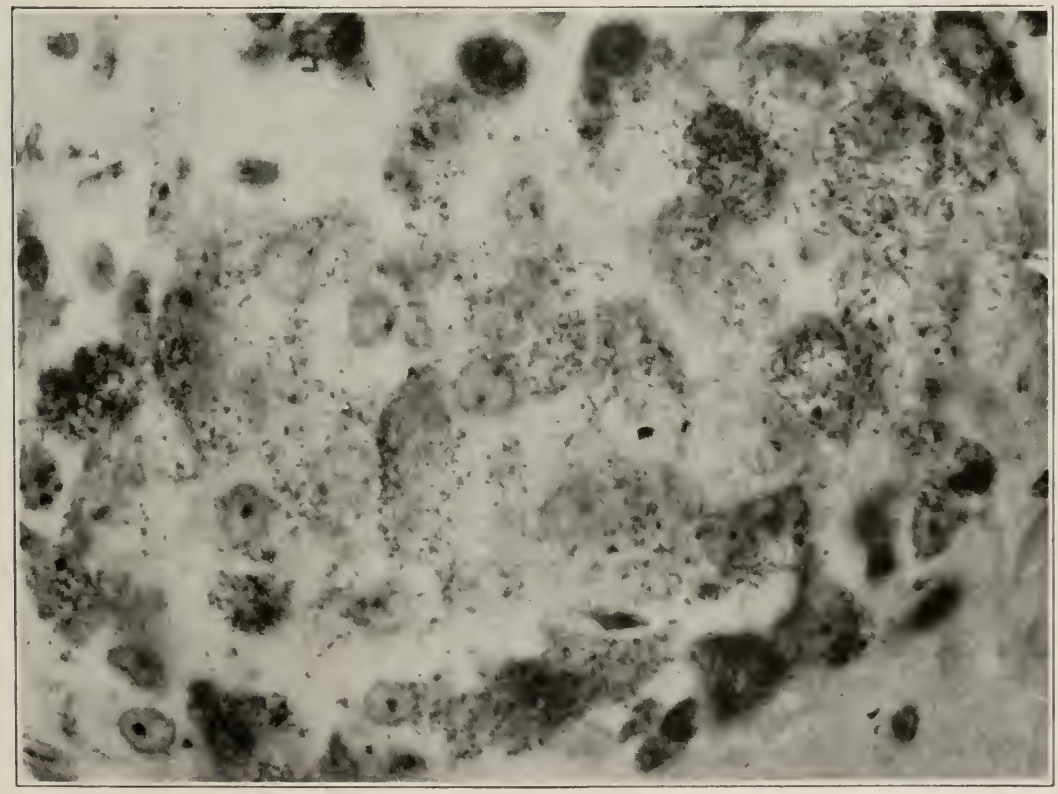

Fig. 4.—Leprosy-like disease in a parrot.

LEPTOTHRIX ON THE CONJUNCTIVA AND IN THE MEIBOMIAN GLANDS

S. R. Gifford

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Case 1 concerns a girl of 22, who had been suffering with recurring attacks of conjunctivitis in both eyes for over a year. She showed, when first seen, a fairly severe conjunctivitis of the right eye, with some sticky secretion, thickening of the lower fold and marked congestion of the bulbar conjunctiva, and of the lid borders, extending along the meibomian glands. The right eye showed blepharitis with congestion of the lid borders and lower fold. The marked involvement of the bulbar conjunctiva and the inflammation along the meibomian glands made this appear somewhat different from the usual case of chronic conjunctivitis. A small amount of secretion was squeezed out of these glands in both eyes and smears and cultures made of it and of the secretion in the lower fold. A zinc collyrium and yellow oxid of mercury ointment were prescribed, and the patient was allowed to return to her home. Five weeks later she wrote that both eyes were improved and that there was no more discharge.

Smears of the secretion in the right lower fold showed numerous gram-positive thread-like bacilli, many 10-15 mikrons long by 1-1¼ thick. Some were curved, and many presented clear refractile subterminal bodies which appeared to be spores (fig. 1). The organisms were all extracellular. Smears from the meibomian secretion showed no organisms.

Inoculation on blood serum from the secretion in the right lower fold gave in 24 hours a pure culture of a gram-positive organism, appearing as long, unbranched, curved threads, with some shorter rods. All forms were nonmotile. In cultures a few days old, coils of the threadlike organism were found 50-100 mikrons long. Spores were formed after 48 hours, seen subterminally on the rods and all along the threads, and taking Moeller's spore-stain. Some free spores were seen. No branching was observed. The serum was liquefied after 7-10 days.

Agar: Growth was best at 37 C., slight at room temperature. At 37 C. a spreading, grayish-white growth appeared in 24 hours, with a slightly feathery edge. No pigment was formed. The growth became slightly striated and roughened, but never showed the folded pellicle of the subtilis group. Cultures were made from single colonies on a plate and showed both short rods and thread forms (Figs. 2, 3 and 4).

Broth: A characteristic growth appeared. The broth remained clear, with no pellicle, while a feathery clump of growth accumulated slowly in the bottom, sending upward long threads that almost reached the surface after 10 days or more.

The milk was decolorized without acid-formation in 48 hours. Later there was peptonization; there was no coagulation.

Potato gave slow, invisible, slightly moist growth.

Gelatin was liquefied slowly around the upper part of the stab, at room temperature.

No odor developed. No gas was formed in sugar broth. The iodin reaction was negative in organisms from all mediums. Spores were most numerous on solid mediums, while the longest threads were found in liquid mediums. There was no growth anaerobically.

Cultures left in the boiling water bath for one hour were capable of growth. Cultures left at room-temperature were viable after several months.

Animal Experiments.—Five minims of washings from an agar slant were injected intraperitoneally in a guinea-pig, which was found dead next morning. Smears from the exudate in the peritoneal cavity and from the omentum showed curved gram-positive organisms, 7-15 mikrons long, nearly all extracellular.

Three cultures from the peritoneal cavity showed the thread-forming organism in pure culture. The heart blood was sterile.

A second guinea-pig was injected subconjunctivally with 2 minims of the same suspension; it died in 23 hours; there was no reaction in the orbital tissues, but subscapular hemorrhages in the kidneys and enlarged spleen. Smears of the orbital tissues showed the characteristic organisms and cultures of the conjunctival sac, orbital tissues and heart blood showed the thread-forming organism. The culture of the orbital tissues contained also a staphylococcus, but the others showed the organism in pure culture.

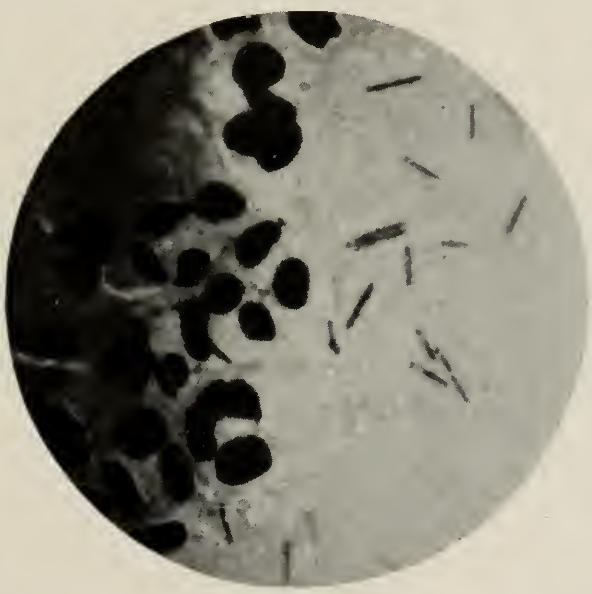

Fig. 1 (Case 1).—Smear of conjunctival sac; × 1200.

Two drops of peritoneal fluid from the first guinea-pig were placed in the conjunctival sac of another pig, and the conjunctiva deeply scarified. It died the following night, and culture from the conjunctival sac showed some growth of the thread former, but this was overgrown by staphylococci. Only a small amount of heart blood was obtained and the culture was negative. Culture of the peritoneal sac gave staphylococci only.

Three other guinea-pigs died 5 to 10 days after one or more smaller injections of cultures, but the organisms were not isolated.

Two intraperitoneal injections in white mice were without effect.

Of two inoculations with loopfuls of growth in my own conjunctival sac, one produced only lacrimation and irritation lasting 24 hours, perhaps from the foreign material; the other was without effect.

Small injections subcutaneously in rabbits were without effect, and two rabbits were subsequently immunized with increasing intravenous doses, without mishap.

Ten day broth cultures were filtered through a No. 10 Chamberlain filter, but the filtrate was without effect on guinea-pigs in doses of 1 c c given intraperitoneally.

Of two serums produced in rabbits, one agglutinated the homologous organism strongly at 1 : 160, less strongly at 1 : 320. The other agglutinated it strongly at 1 : 320, less strongly at 1 : 640. The organism was not agglutinated in any of the controls of normal salt, normal rabbit serum, or normal human serum. Neither serum agglutinated the somewhat similar organism from case 2 nor a similar thread-forming organism obtained as a contaminant on old serum. A strain of B. subtilis was tested with serum 1, with negative results in all dilutions.

Case 2 concerns a man of 27, who had symptoms suggesting eye strain for the past year. He was found to have a slight refractive error, but the congestion of the conjunctiva, especially on the tarsi in the region of the meibomian glands combined with a small amount of sticky secretion in the sac, seemed to

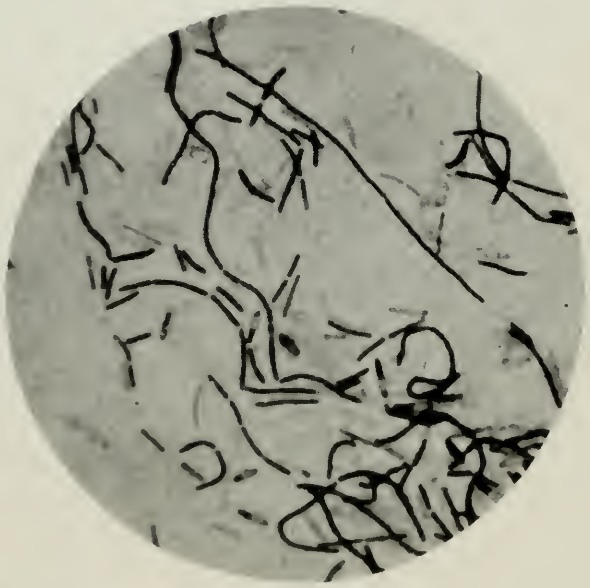

Fig. 2 (Case 1). Smear of agar culture, × 1200.

indicate another factor to account for it. No organisms were found in a smear of the conjunctival secretion. Pressure on the meibomian glands expressed an excess of whitish waxy, semifluid material from all their orifices.

A zinc collyrium was prescribed. The symptoms were relieved for a time, but in two weeks the patient returned with the same trouble. A correction for reading was given, and the glands thoroughly squeezed out again. This was repeated in 6 weeks; there was still an excess of fluid, though the symptoms were slight and the congestion much less.

Smears of the meibomian secretion showed a fair number of large bacilli with rounded ends. There were no true threads, but some of the bacilli were as long as a hay-bacillus, and all were much thicker than B. xerosis. Their reaction to Gram's stain varied, both gram-negative and gram-positive organisms of the same morphology being seen. Staphylococci were also found.

A broth culture showed a predominance of long gram-positive bacilli, some 10-12 mikrons in length, and often curved. Staphylococci and xerosis bacilli

were also present. A pure culture of the long gram-positive organism was obtained from a plate, and after 2 to 4 days' growths many threads 10-20 mikrons long were found and some 20-50 mikrons long. Some of these became clubbed at the ends, and subterminal spores were formed, which stained by Moeller's spore-stain. No branching was observed. The organism was non-motile (Fig. 5).

Growth on agar at 37 C. appeared as a delicate grayish film, spreading over the medium, but not showing the wrinkling and filaments of the subtilis group.

Milk was slightly acidified but not coagulated. No pellicle was formed in broth. Blood serum was not liquefied. There was no growth at room temperature. The longest threads were formed in fluid medium.

A culture left in the boiling water broth for 12 minutes was capable of sub-culture. After 24 minutes it was no longer viable.

The growths from a 6-day agar slant injected intraperitoneally into a guinea-pig produced no effects. Scarifying the conjunctiva and rubbing in a

Fig. 3 (Case 1).—Smear of agar culture, showing spores in threads, × 1200.

loop of growth produced no effects. Injection into the vitreous of a guinea-pig was negative.

Agglutination with serum I prepared against the organism from case 1 was negative. Whether the large bacillus in the smears was the same organism isolated in culture is questionable. The thread-formation in culture, however, was only marked after 2-4 days, so it is possible that the organism might not form threads on its human host and still form them in medium that favored complete development. The organism in the cultures, by its unbranched thread-formation, is seen to be a Leptothrix, and whether a pathogenic agent or not in this case, at least an inhabitant of the meibomian glands or lid-border.

Case 3 concerns a man of 60, who complained of a burning sensation and tired feeling in his eyes for over a year. Vision was brought up to 20/20, both eyes, with suitable correction. Both eyes were inflamed, with lids thickened and reddened at the ciliary margin. On squeezing the lids, a large amount of semitransparent honey-like fluid was expressed from most of the meibomian

glands. Smears and a culture were made of this. As the patient was obliged to return home at once, the lids were squeezed out as completely as possible, zinc chlorid 1 grain to the ounce was prescribed with zinc cerate ointment for the lids, and his physician was instructed how to express the fluid from the lids twice a week. A letter two months later reported no further symptoms while the treatment was continued.

The smears showed few organisms. After a long search, an area was found showing a fair number of curved, gram-positive threads, some 20-25 mikrons

Fig. 4 (Case 1).—Four-day agar culture.

long, with shorter, gram-positive rods, some of which were curved (figs. 6 and 7). No branching was seen. A few gram-positive threads were found in other smears, also a few gram-positive diplococci. It seemed as if a small lump or concretion of the thread-like organisms had been broken up on the smear, while no such concretions had been obtained on the others.

An aerobic culture on blood serum was the only one taken and this showed staphylococci and xerosis bacilli. From the smears alone, a positive diagnosis between Leptothrix and Streptothrix can hardly be made, since it is known that Streptothrix may show branching only in culture. The threads were 1-1¼ mikrons wide, however, about twice as wide as the threads of most strepto-

thrices, and showed refractile sporelike bodies similar to those seen in the smears of case 1, so that the diagnosis of Leptothrix may be considered the most probable.

Smears from the secretion in two other cases of chronic meibomitis in which repeated examinations were made have shown a few large gram-positive bacilli, up to 12 mikrons long and sometimes curved. These were always associated

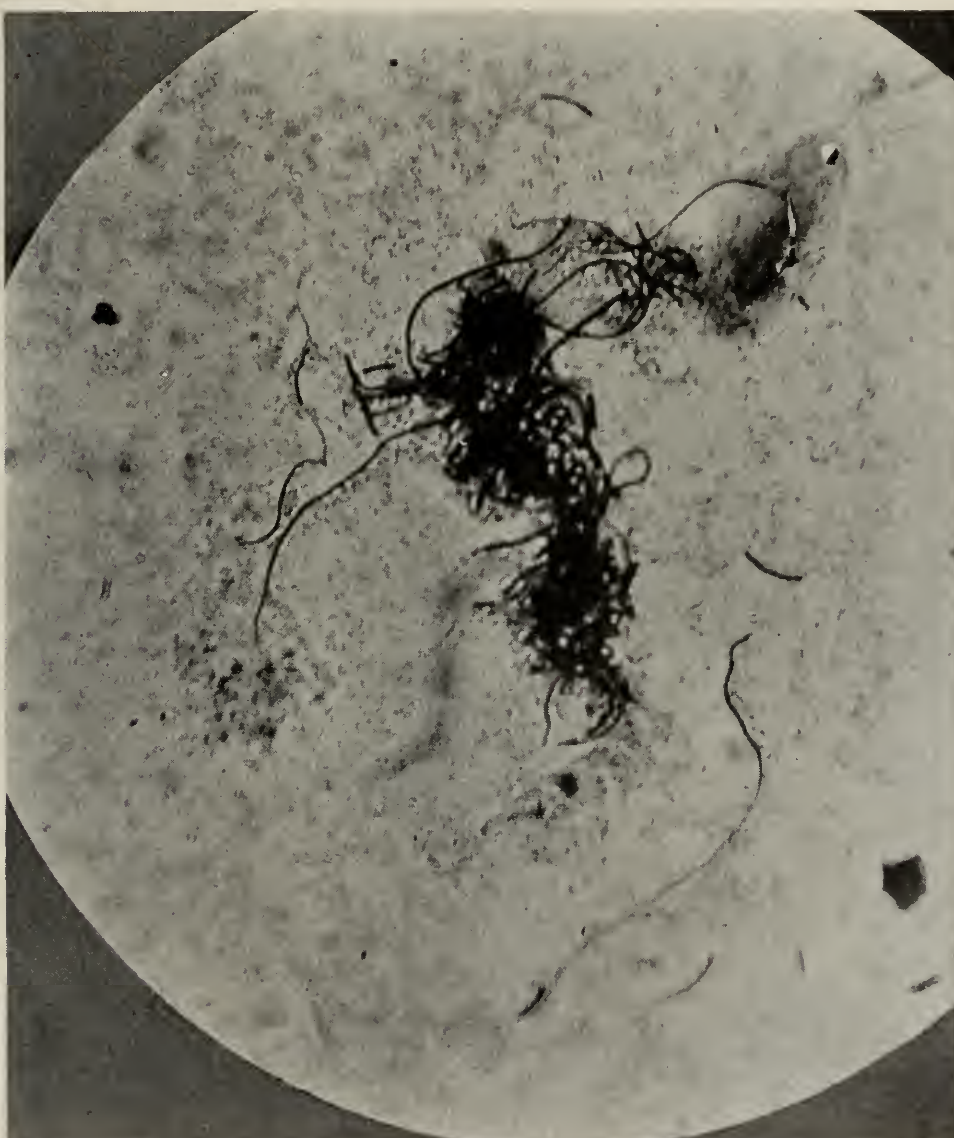

Fig. 5 (Case 2).—Smear of 48-hour culture.

with staphylococci and xerosis bacilli in the smears, and no pure cultures of organisms resembling the large bacilli have been obtained; therefore their identity is questionable.

Since the early description of Leptothrix by Robin in 1847[1] there has been some confusion as to the classification of this organism, owing chiefly to the

[1] Quoted in Klebs: Eulenberg's Encyclopedia, 8, p. 260.

fact that it has been so rarely grown in pure culture. Probably the most satisfactory classification of the higher bacteria is that of Petruschky,[2] who divides the Hypomycetes into true molds and Trichomycetes; the latter is made to include, as four distinct groups, Leptothrix, Cladothrix, Streptothrix, and Actinomyces. The Leptothrix group is characterized by its formation of unbranched threads. The iodin-reaction, as a mode of distinction between Leptothrix and Streptothrix, may be considered as of no value, since Fricker[3] and others have shown that it is due to the presence or absence of starch granules, which depends entirely on the previous nutriment of the organism.

The occurrence of Leptothrix in the body as a saprophyte has been well known since Bizzozero[4] described Leptothrix epidermidis as a constant finding on the skin and Miller[5] described four types of Leptothrix buccalis in the normal mouth.

Fig. 6 (Case 3). Smear of meibomian secretion.

A number of authors have found it in smears of various lesions, usually of the pharynx and respiratory tract. Leyden and Jaffé[6] found masses of unbranched threads and spores in the plugs of sputum in putrid bronchitis. Introduction of these plugs by tracheotomy into rabbits produced fatal bronchopneumonia in some cases; the organisms were found in sections. Feeding the plugs produced bloody diarrhea in rabbits. These authors found similar threads in decubitus ulcers, gangrenous wounds, the feces of cholera patients and in a closed abscess of the tongue; in the latter they formed small, hard concretions. In mycoses of the mouth Leptothrix has been found by Michelson,[7] Fraenkel,[8]

[2] Kolle u. Wass. Hdbch., 1903, 2, p. 832.
[3] Zentralbl. f. Bakteriol., 1904, 36, p. 369.
[4] Handbuch der klin. Mik., 1887; Virchow's Arch., 1884, 98, p. 441.
[5] Bakteriologie der Mundhöhle, 1894.
[6] Deutsch. Arch. f. klin. Med., 1867, 2, p. 488.
[7] Berl. klin. Wchnschr., 1889, 9, p. 284.
[8] Quoted by Petruschky.[2]

Chiari,[9] Stoos,[10] Dubler,[11] Hering,[12] Councilman, Malloy and Pearce,[13] Epstein,[14] Wright,[15] Newcomb[16] and others.

Dubler's patient died of bronchopneumonia, and white flecks consisting of Leptothrix masses were found on the mucosa of the mouth, esophagus and larynx. Sections showed the organisms under the epithelium, in the glands and in the lymph nodes.

Hering's findings were in the tonsillar crypts of 6 cases. He inoculated rabbits with the plugs subcutaneously, and produced small abscesses, from which however the organisms were not recovered. Newcomb found Leptothrix in two types of cases: first, in cases of folliculosis lacunaris, in which the organism was purely saprophytic, and second, in true mycoses caused, he says, by the Leptothrix.

Von Arx,[17] besides one case of tonsillitis, found the organism in two cases of phlegmon of the neck, one associated with carious teeth, but in all the other

Fig. 7 (Case 3).—Smear of meibomian secretion showing spores.

cases mouth organisms were also present and only a few threads were grown in mixed culture. He mentions a similar case of Niehaus, with positive cultures, but it seems doubtful from his description that the organism recovered was a Leptothrix. Injection of the pus subcutaneously killed a guinea-pig, but a mixed culture was recovered. Vaginitis, in which Leptothrix was found, is reported by von Herff.[18] Pearce[19] found it in smears and sections from a case

9 Rev. Mens. de Laryngol., Otol. & Rhinol., 1887, 7, p. 559.
10 Quoted by Petruschky.
11 Virchow's Arch., 1891, 136, p. 454.
12 Ztschr. f. klin. Med., 1883. 7, p. 358.
13 Councilman, Mallory and Pearce: Quoted by Pearce.[19]
14 Prag. med. Wchnschr., 1900, 15, p. 253.
15 Laryngoscope, 1898, 4, p. 221.
16 Laryngoscope, 1898 4, p. 246.
17 Correspbl. f. Schweizer Aertze, 1889, p. 161.
18 Sammlung klin. Vorträge, 1895, 137.
19 Univ. of Penn. Med. Bull., 1901, 14, p. 217.

of necrosis of the larynx perforating into the esophagus and in the biliary ducts of a patient dying after an operation for gallstones. Animal inoculation was negative. Naunyn's [20] finding of Leptothrix on the pia mater and vegetations in the heart of a patient with chorea is doubtful because the organs had been washed in tap water which contained similar organisms. Majocchi,[21] in a series of concretions of the salivary ducts, found that some were caused by each of three organisms: Leptothrix, Streptothrix Foersteri, and Actinomyces. Leber [22] repeatedly inoculated rabbits' corneas with fresh material from the tonsils containing Leptothrix, and observed progressive lesions of the cornea, sections of which showed Leptothrix elements. He appears not to have worked with pure cultures.

It is only necessary to mention briefly the discussion among dental pathologists centering around the work of Vincentini.[23] This author ascribed a large number of our ills to infection by forms of Leptothrix, but the elaborate life cycle of the organism he described places it definitely outside the group of leptothrices that other observers have studied in culture and among those higher moulds that some botanists classify as Leptothrix.

In the ophthalmologic literature, a large number of concretions of the canaliculi purporting to be formed by Leptothrix have been reported. Most of these were studied in smears and secretions only, and it is the opinion of Axenfeld [24] and others who have studied cultures that nearly all such concretions are due to a Streptothrix; Streptothrix foersteri or Streptothrix actinomyces (the author has studied 5 such concretions, in 2 of which pure cultures were isolated, and all showed the branching threads of Streptothrix). Fifteen observers of these concretions called the organisms found Leptothrix, but since the branches of Streptothrix may be so small as hardly to be noticed in smears or sections, Axenfeld justly concludes that a diagnosis of the Leptothrix is not justifiable without cultures. Of this group, Cannas [25] alone obtained Leptothrix in cultures; and this under somewhat doubtful circumstances. Thus, though some of these cases may have concerned Leptothrix, this cannot be said to have been proved except possibly in the case of Cannas. Verhoeff and Derby [26] and Keiper [27] found Leptothrix in sections of excised conjunctiva and of preauricular glands in Parinaud's conjunctivitis, but these observations are open to the same objection, no cultures being obtained and sections being, in some ways, even more unsatisfactory than smears for the study of threadlike organisms.

The same may be said of the other cases reported above, in which no cultures were obtained. In a few cases, however, cultures of Leptothrix have been definitely successful. I was able to find only two reports of observers who have definitely isolated Leptothrix from lesions and three from normal tissues; also more doubtful cases.

Arustamow,[28] in repeated smears and cultures from the urine of a tabetic, found threads 8-50 mikrons long and 0.5-6 mikrons thick. Pure cultures were obtained with great difficulty, growing slightly and only anaerobically better

[20] Rev. in Baumgarten's Jahresber., 1888, 4, p. 296.
[21] Arch. per le scienze med., 1892, 16, No. 15. (Quoted by Cannas.[25])
[22] Centralbl. f. d. med. Wiss., 1873; Berl. klin. Wchnschr., 1882, p. 161.
[23] Dental Cosmos, 1900, 1901, 1903.
[24] Bacteriology of the Eye, 1908.
[25] Ann. di. Ott., 31, p. 606.
[26] Arch. f. Augenh., 1913, 75, p. 207.
[27] Ophth. Rec., 1914, 28, p. 109.
[28] Centralbl. f. Bakteriol., 1890, 6, p. 349.

in acid mediums. The organism was nonmotile and showed refractile deeply staining bodies in old cultures, which the author thought were probably spores though they were not viable after 3 months. The organism did not grow at room temperature, and did not liquefy gelatin.

From the tonsillar crypts of two cases of tonsillitis he isolated an organism morphologically similar to the first, but growing aerobically very freely, forming a folded gray coat on agar. It formed a heavy pellicle on broth, was nonmotile, grew at room temperature, and liquefied gelatin. It showed also septums in the threads, which the first organism did not show. It showed sporelike bodies, and was viable after three months. Both organisms were only one-third as thick as Vignal's organism and those of Klebs and Rasmussen. Arustamow says that Affanassieff obtained a culture of a similar organism, but no reference to it could be found.

The author reports no animal experiments.

Cozzolino [29] obtained clear-cut and complete results in a case of periauricular swelling in a young girl, who died from a large retropharyngeal abscess and symptoms of basilar meningitis 6 months after her first visit. There was boardlike swelling of the neck, clinically like actinomycosis; but smears and cultures from the periauricular swelling, the retropharyngeal abscess, and the lungs, kidneys, spleen, brain and medulla all showed the same organism, which must be identified as a Leptothrix. Smears showed long coils of gram-positive unbranched threads and some bacilli. Granules found in the pus were composed of a central mass of threads and an outer fringe of threads with clubbed ends.

Thirty pure cultures were obtained from the different organs. On the various mediums the organism behaved somewhat like the organism from the author's case 1, growing slowly aerobically at room temperature, well at 37 degrees and at a temperature as high as 60 C. It liquefied gelatin and serum, precipitated the casein in milk and reduced lacmus. The spokelike bodies found both free and in threads stained only faintly with Moeller's spore stain, but resisted heating to 100 C. for 15 minutes; hence they were evidently spores. No branches were seen in cultures. The organism formed reddish pigment, however, on serum and egg-white and a pellicle on broth; its growth became dry and folded, differing in these particulars from the organism of case 1. The young bacilli in Cozzolino's case also showed rapid independent motility. Its pathogenicity resembled that of this organism, being marked for guinea-pigs and negative for rabbits and white mice. A guinea-pig died 19 hours after subcutaneous injection. The organism was also pathogenic for house mice. Cozzolino called his organism B. filiformis, but it is evidently a form of Leptothrix.

Vignal,[30] in a series of plate cultures from the tartar of normal teeth, isolated a slow-growing aerobic organism, forming unbranched threads 1.6 to 30 mikrons in length. Cultures resembled those from case 1, but no spores were mentioned. Tests for pathogenicity were not described.[31]

Bordoni-Uffreduzzi,[32] from the skin between the toes and in the groin of normal persons and those with intertrigo, isolated a Bacillus epidermis which they say is identical with Bizzozero's Leptothrix epidermis. It grew at room temperature on the surface of agar, serum and potato, forming a wrinkled gray

[29] Ztschr. f. Hyg., 1900, 33, p. 36.

[30] Arch. de Phys., 1886, 8, p. 325.

[31] Though Bizzozero (4) describes completely the morphology of Leptothrix epidermidis as he found it on the normal skin and the skin in intertrigo, I could find no work of his in which he described cultures.

[32] Fort. d. Med., 1886, 4, p. 151.

coat on agar. It gave little growth on gelatin and evidently did not liquefy it. It formed spores, becoming free as the threads broke up. The threads were 0.3 mikron in diameter (much thinner than those of Vignal and the author), and were separate, breaking up into shorter rods.

Inoculations into guinea-pigs, rabbits and the author's own skin were negative.

Lehmann and Neumann [33] evidently grew a Leptothrix from the skin. Their organism formed unbranched septate threads, grew freely on the usual mediums aerobically, liquefied gelatin and was gram-positive. It differed from the author's organism in forming brownish-yellow pigment, a pellicle on broth and milk, folded coat on agar, in that the young rods showed active motility and in the absence of spores.

Reports with positive culture in which the diagnosis was more doubtful are the following: Flexner [34] isolated an extremely pathogenic organism from the uterus, serous cavities and viscera of a rabbit dying 5 days after abortion. A dense network of threads 1.4 to 154 mikrons long was found to cover all the serous surfaces. The organism showed no growth on ordinary mediums and was only grown on sterile rabbit tissue. Inoculations from the tissues and from these tissue-cultures caused fatal peritonitis, meningitis, abortion and septicemia in rabbits and guinea-pigs, but were negative in mice, pigeons and one dog. The organism is described as gram-negative, though with deeper staining dots, globules and cylinders within the threads. It often showed a grouping in sheafs, radiating from an apex or in rosettes, in which it resembles cladothrix. It formed no spores, as it was killed by drying and heating to 55 C. It showed no branches. Flexner called it Bacillus (Leptothrix?) pyogenes filiformis. Follet and Sacquepee [35] isolated what they called Leptothrix from the pustules of a case of herpes zoster of the abdomen. The organisms grew on ordinary mediums and formed long threads. The threads, however, showed frequent false branding. The shorter forms were actively motile. No spores were described. The organism produced nonfatal peritonitis in guinea-pigs. The organism isolated by von Dobrzyniecki [36] from a root-filling removed from a tooth is called by him Leptothrix placoides alba, and was grown in pure culture. His pictures of it, however, show what appears to be true branching. Rosenbach [37] isolated from the lesions of a dermatitis common among fish-mongers, a threadlike organism which showed false branching only, and which he thinks is a species of Cladothrix. It formed clubshaped terminal bodies thought to be spores. It grew on gelatin, and cultures produced similar lesions on the author's own arm.' Schmorl,[38] from an infectious disease of rabbits starting in the lips and producing local necrosis and later peritonitis, obtained a thread organism that grew only on anaerobic blood serum. Cultures produced similar lesions in rabbits, and were pathogenic for white mice, but not for other animals. No branches were described, and the organism is classed as a Leptothrix or a Cladothrix (the original reference was not in the Surgeon-General's Library). Jacobson [39] grew a threadlike organism without branches from the tonsillar crypts, but describes no single colonies on plates, and the reviewer doubts that a pure culture was obtained. Arustamow considers his cultures an

[33] Bakteriol. Diag. 1896, p. 395.
[34] Jour. Exper. Med., 1896, 1, p. 211.
[35] Bull. d. hôp. de Par., 1902, 7, p. 568.
[36] Centralbl. f. Bakteriol., 1897, 21, p. 225.
[37] Arch. f. klin. Chir., 1887, 36, p. 346.
[38] Ztschr. f. Thiermed., 1891, p. 375. Rev. in Baumgarten's Jahresbericht, 1893, 9. p. 7.
[39] Rev. in Baumgarten's Jahresbericht, 1888, 4, p. 283.

organism from poorly sterilized potato. Cannas,[25] as mentioned above, grew from a lacrimal concretion an organism forming unbranched threads. It grew easily on ordinary mediums when once isolated, and was evidently a Leptothrix. However, since only one colony of it grew from the original material, other organisms being present, Axenfeld doubts whether it was the responsible organism.

SUMMARY

It seems fairly certain that there are several distinct organisms within the group of Leptothrix. Miller's distinction of four species of Leptothrix buccalis on the basis of morphology in smears and the iodin reaction is, however, unsatisfactory. There seems to be no biologic distinction between Leptothrix epidermidis and Leptothrix buccalis, organisms from both the skin and the mouth probably falling in one of several species.

The only definite evidence of such grouping must be obtained from the few strains grown in pure culture. Properties of such organisms, about which sufficient data were given to justify their being called leptothrices, are summarized in table 1.

This shows differences in properties, of more or less importance in all the strains for which the data are at all complete. A few common properties may be noted. All were aerobes, except Arustamow's strain 1. All were gram-positive (when this was mentioned) except Flexner's organism. All were nonmotile, except those of Cozzolino and Lehmann and Neumann. Those that grew freely at room temperature, all liquefied gelatin except the strain of Bordoni-Uffreduzzi and the author's strain 2. None formed pigment except those of Cozzolino and Lehmann and Neumann, which agree more nearly than any other two, both being motile and forming pigment. The author's strain 1 was much like these two except in the last two respects and in its not forming pellicle on broth. Of the author's two strains, 1 grew freely even at room temperature, formed long threads with many free spores and no clubbed ends, and was definitely pathogenic; while 2 grew delicately, formed shorter threads with fewer spores, showed threads with clubbed ends, and was nonpathogenic for animals. Serologically 1 showed no relation to 2. Arustamow's strain 1 and Flexner's organism are each quite different from all the others.

Out of such a confusion of small differences, it would be useless to attempt a definite classification. The property of spore-bearing, however, seems to the author to be less subject to special conditions than most of the properties enumerated, and in this case more than a matter of one or two exceptions. The author's two strains, Cozzolino's

PROPERTIES OF LEPTOTHRICES GROWN IN PURE CULTURE

Author	Source	Gram Stain	Spore Forma-tino	Iodin Reac-tion	Motility	Oxygen Require-ments	Liquefac-tion of Gelatin	Pellicle on Broth	Pigment Forma-tion	Pathogenicity
Arustamow 1............	Urine of a tabetic.........	No mention	Probably +	No mention	—	Annerobe	—	—	—	Not tested
Arustamow 2 (2 strains)...	Tonsillar crypts in tonsillitis	No mention	Probably +	No mentio n	—	Aerobe	+	+	—	Not tested
Cozzolino..............	Periauricular abscess and viscera at postmortem	+	+	No mention	+	Aerobe	+	+	Red on serum and egg white	Positive for guinea-pigs and house mice; negative for rabbits and white mice
Vignal..................	Tartar of normal teeth....	No mention	—	No mention	No mention	Aerobe	+	No mention	—	Not tested
Bordoni-Uffreduzzi...	Normal skin and skin in intertrigo	No mention	+	No mention	No mention	Aerobic	—	No cention	—	Negative; guinea-pigs, rabbits and human skin
Lehmann and Neumann...	Normal skin..............	+	—	—	+	Aerobe	+	+	Agar: brownish yellow; potato, reddish brown	Not tested
Flexner..................	Uterus and viscera of rabbit at postmortem	—	—	—	—	Aerobe (only on tissues)	—	—	—	Positive for rabbits and guinea-pigs; negative for mice, pigeons and dogs
Cannas..................	Concretions of canaliculus	+	—	+	No mention	Aerobe	+	—	—	Negative
Author 1................	Conjunctiva in recurring conjunctivitis	+	+	—	—	Aerobe	+	∓	—	Positive: guinea-pigs; negative: rabbits, white mice, author's conjunctiva
Author 2....	Meibomian glands in chron'c meibomitis	+	+	—	—	Aerobe	—	—	—	Negative: guinea-pigs and author's conjunctiva

strain and the skin strain of Bordoni-Uffreduzzi, showed definite spore-formation, and Arustamow's strains with the author's case 3 showed what were probably spores. Those of Flexner, Lehmann and Neumann, Vignal and Cannas, showed none. It might be worth while to place the first mentioned organisms in a tentative group of Leptothrix sporogenes, calling the others Leptothrix simplex. As more pure cultures are reported, these groups will probably be found to contain several distinct species. It may be found, however, that all forms may produce spores under certain conditions.

There can be no doubt, especially in view of Cozzolino's results, which are borne out by the author's and probably also by those of Flexner, that true leptothrices can be distinctly pathogenic for man and animals. It seems likely that many of the reports of Leptothrix in smears alone, as a pathogenic organism, especially in the upper respiratory tract, really did concern Leptothrix. The finding of Leptothrix in the ocular apparatus makes it more probable that some of the cases of lacrimal concretions said to have been caused by Leptothrix, really were caused by that organism and not by Streptothrix, though Streptothrix is undoubtedly much the commoner agent.

Whether there are fast pathogenic strains of Leptothrix or whether the ordinary Leptothrix of the mouth and skin becomes pathogenic under certain conditions cannot be stated. The most likely source of Leptothrix in the eye would seem to be from the mouth by way of a finger, though it may prove to migrate occasionally from the skin of the lids into the meibomian or other glands and thence to the conjunctival sac.

CONCLUSIONS

A strain of Leptothrix was found as the only organism in smears and cultures from the conjunctival sac in a case of recurrent conjunctivitis. It was pathogenic for guinea-pigs and showed definite spore-formation.

A Leptothrix was isolated from a case of chronic meibomitis. It showed definite spore-formation, but was nonpathogenic for guinea-pigs, serologically distinct from the first strain, and presented cultural differences from it.

What is probably a Leptothrix was found in smears from a second case of chronic meibomitis. It apparently showed true spores.

The group of leptothrices includes at least two, and probably more, distinct species.

A RAPID METHOD OF PNEUMOCOCCUS TYPING

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Because of its prognostic value and also because it is necessary for specific serum therapy, a number of methods for the determination of pneumococcus types have come into use.

The standard methods up to 1917 are reviewed by Blake,[1] special emphasis being placed on intraperitoneal inoculation of the mouse with washed sputum and agglutinin and precipitin tests of the peritoneal exudate. Avery[2] reports a rapid cultural method for the determination of pneumococcus types in lobar pneumonia. By the use of a meat infusion broth with 1% glucose and 5% rabbit blood, sufficient growth usually is obtained within 5 or 6 hours for precipitin test made with the clear fluid.

In order to still further save time, Mitchell and Muns[3] describe a method for detecting pneumococcus precipitinogen in sputum, 5 c c of which are ground in a small mortar with sand until a paste is formed. Then 10 c c of normal salt solution are slowly added and stirred into the mixture and after three or four minutes, the dissolved sputum is pipetted off, the solution centrifugalized at 2,200 revolutions per minute for from 5 to 10 minutes, and a precipitin test made with the clear fluid.

Krumweide and Valentine[4] suggested a coagulation method for the rapid determination of precipitable substances in the sputum. As in the method of Mitchell and Muns, considerable quantities of sputum are required, a decided objection against the method in certain cases in which only small amounts of sputum can be obtained. From 3-10 c c of sputum in a test tube are placed in boiling water until a "more or less firm coagulum results. The coagulum is then broken up with a heavy platinum wire or glass rod and saline is added. Just enough saline should be added so that, on subsequent centrifuging, there will be sufficient fluid to carry out the test." The tube is again placed in boiling water for several minutes, after which centrifugalization is employed. The supernatant fluid, which is the antigen, is then floated over 0.2 c c of undiluted antiserum. "If a fixed type was present in the sputum, and should the sputum have been rich in antigen, a definite contact ring is seen in the tube containing the homologous serum. With sputums less rich in antigen, the ring may develop more slowly and it will be less marked."

The test that I describe is based on the solubility of the pneumococcus in bile. Taking advantage of the fact that in a typical case of lobar pneumonia the infecting type of pneumococcus is often found

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[1] Jour. Exper. Med. 1917, 26, p. 67.

[2] Jour. Am. Med. Assn., 1918, 70, p. 17.

[3] Jour. Med. Res., 1917-8, 37, p. 339.

[4] Park and Williams, Pathogenic Micro-organisms, 1920, p. 318.

in predominating numbers in the sputum of the patient, 1 cc. or less of such sputum, immediately on its receipt in the laboratory, is stirred in sterile salt solution and bile added. After the protein has gone into solution in the bile, the mixture is filtered and a precipitin test is immediately made with the filtrate. The series on which this test was used comprises 25 cases. On an average, the time for effecting a typing was from 20 to 30 minutes. The results follow:

Methods Employed and Pneumococcus Types

Cases	Pneumococcus Types		
	Rapid Precipitin	Avery's Method	Mouse
1	3	3	—
2	2	2	—
3	1	1	—
4	2	2	—
5	Negative	Negative (streptococcus)	Negative (streptococcus)
6	3	3	—
7	1	1	—
8	2	2	—
9	1	1	—
10	Negative	4	4
11	Negative	4	4
12	Negative	4	4
13	Negative	4	4
14	Negative	4	4
15	3	3	3
16	1	1	1
17	Negative	4	—
18	Negative	4	—
19	Negative	4	—
20	Negative	4	—
21	Negative	Negative (streptococcus)	—
22	Negative	4	4
23	Negative	4	4
24	Negative	Negative (streptococcus)	Negative (streptococcus)
25	Negative	Negative (streptococcus)	Negative (streptococcus)

We note that 4 cases belong to type 1, 3 to type 2, 3 to type 3, and 11 to type 4.

A direct smear of the sputum, a selected fragment of sputum being chosen, is stained by Gram's method, as examination of such a smear is of distinct value in determining the presence of the pneumococcus as well as its relative numbers in the sputum. Next 1-1.5 cm. of sputum are placed in a clean test tube which contains a glass rod. Normal salt solution is then added, small quantities (0.1 to 0.2 c.c.) being added at a time and vigorous stirring with the glass rod following the addition of each portion of solution. After from 0.5 to 0.8 c.c. of salt solution have been stirred into the sputum, from 3 to 5 drops of undiluted ox bile are added and the mixture thoroughly stirred. The amount of salt solution to be added is dependent on the

consistency of the sputum, the endeavor being to obtain a fairly homogeneous specimen of a sufficiently fluid nature to admit of filtration or centrifugation. The tube is then immediately placed in a water bath, in water at 45 to 48 C. for 10 to 20 minutes, which suffices for solution of the pneumococci by the bile. The fluid is then immediately filtered. For filtration, a filter paper listed as "Eimer and Amend Best White Filter Paper No. 15" has been employed. The filter paper mounted preferably in a small funnel having a long stem, is first moistened with a small amount of normal salt solution. Filtration at ordinary atmospheric pressure will usually be somewhat slow. By the use of a suction pump and a small amount of negative pressure, the process of filtration is greatly facilitated. It has been my experience that the filtrate so obtained is clear and colored to only a slight extent by the bile.

In lieu of filtration, centrifugilization may be employed. After the pneumococcus protein has dissolved in the bile, the mixture is placed in a centrifuge tube and a small amount of cotton, with the fibers loosely united, is placed on top of the fluid. Centrifugalization is commenced at low speed and the speed is gradually increased up to about 2,000 revolutions per minute. As the speed increases the cotton is pulled down to the bottom and assists appreciably in clearing the solution.

Of the filtrate or centrifugate, 0.3 to 0.5 cm. are now pipetted into each of three small tubes. To the first tube is added one drop of undiluted type 1 pneumococcus antiserum, to the second tube is added one drop of undiluted type 2 antiserum and an equal quantity of type 3 antiserum is added to the third tube. In case of a doubtful reaction, the addition of another drop of antiserum may be indicated.

When a positive precipitin test is obtained, a clouding occurs in the fluid almost immediately on the addition of the specific antiserum. The test becomes still more marked if the tubes are immersed for from 10 to 20 minutes in water at 48 C. Following this, if the tubes are placed in the icebox, the positive tube, after several hours, will show a sedimentation of the specific pneumococcus proteid, the supernatant fluid appearing clear.

In all of the cases in which a positive precipitin test for one of the first three types of pneumococci was obtained, the results were identical with those obtained by the Avery method. In 5 instances, the sputum of the same patient was typed on several consecutive days,

with identical results, in each case the rapid precipitin test checking with the Avery method. In the 15 cases in which the rapid precipitin test was negative, the Avery method revealed a type 4 pneumococcus in 11 of the series, and in the remaining 4 cases, a streptococccus. In this series of 15 negative tests, intraperitoneal inoculation of mice with washed sputum was employed in 10 and in each instance the results were the same as those obtained by the Avery method.

The necessity of obtaining a true specimen of sputum from the deeper air passages as free as possible from saliva was strikingly brought out in case 8. The first specimen was not sputum, but saliva, and the rapid precipitin test and the Avery method showed a type 4 pneumococcus (probably from the mouth). A blood culture the following day revealed a type 1 pneumococcus.

In the cases, the parallelism between the results of the rapid precipitin test and the Avery method is striking. It would seem to suggest that when a typical pneumonia sputum is received on which the rapid precipitin test fails to reveal a "type," immediate intraperitoneal injection of a white mouse with the washed sputum would be indicated. On the other hand, when the rapid precipitin test is positive, it would seem justifiable, so far as we have gone, to accept the results for clinical purposes, especially in cases in which the rapid precipitin test reveals a type 1 pneumococcus, in which case immediate administration of type 1 antiserum would be indicated.

Two of the patients in whom the rapid precipitin revealed a type 1 pneumococcus were exceedingly ill on admission and in both instances the sputum was "typed" within half an hour. In both instances the prompt administration of type 1 pneumococcus antiserum was succeeded by recovery.

Microscopic examination of direct smears of the sputum is of distinct value in that it gives an idea of the relative numbers of pneumococci in the sputum. The greater the number of pneumococci in a given sputum, if they be of one of the first three types, the greater will be the amount of pneumococcus protein dissolved by the bile and the more rapid and clean-cut will be the precipitin test obtained on addition of the specific antiserum. Hence the sputum chosen for the test should be that portion which contains pneumococci in the largest numbers, this being usually the portion which is most streaked with blood or most purulent.

SUMMARY

The method described is a rapid precipitin test of filtered pneumonic sputum, to which bile previously has been added. By this method a "typing" of the pneumococcus may be effected within half an hour after receipt of the sputum.

INHALATION EXPERIMENTS ON INFLUENZA AND PNEUMONIA, AND ON THE IMPORTANCE OF SPRAY-BORNE BACTERIA IN RESPIRATORY INFECTIONS

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While the influenza virus appeared to be of reduced virulence during the outbreak of February and March, 1920, the occasional occurrence of a family outbreak, or of rapid death due to pulmonary edema, seemed to indicate that it was identical with that present in the outbreak of 1919. We feel particularly sure that we were dealing with cases of influenza in the family S., because the mother, father and eight children all came into the hospital at once. Most of these ten patients had bronchopneumonia and one purulent pleuritis. Cases R. and S., B. and B. and T. were of a milder type, although B. and T. had secondary bronchopneumonia. These cases were chosen because most of them had been ill for only a day or two at the time the material was collected.

Thirty-eight cases, including those just cited, were examined bacteriologically. All aerobic, partial tension and anaerobic blood cultures were negative. For the throat and sputum cultures we used + 0.5 agar containing 5% of rabbit blood. Incubation was at 37 C. under aerobic, partial tension and anaerobic conditions. B. influenzae was isolated from 12 cases. Six of the 12 were from the family S. who all became ill at once. From the father of this family we failed to isolate B. influenzae, and he was ill for months with streptococcus empyema. In 6 cases B. influenzae was the predominating organism and in 4 it was present in pure culture.

When B. influenzae was grown at partial tension it retained its minute bipolar form and showed less tendency to involution than when grown aerobically. All the strains were strictly hemoglobinophilic while the Koch-Weeks bacillus was isolated on aerobic, partial tension and anaerobic slants of ascites agar, and could be subcultured

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on this medium. All strains failed to produce indol in hemoglobin broth when the sulphuric acid-sodium nitrite test was applied. However, the growth in our broth was scanty. Most of the associated bacteria belonged to the pneumococcus, hemolytic streptococcus and staphylococcus groups. No colonies that might have represented the B. enteritidis (type M 5), which appeared in the sprayed animals, were noted, and it is not likely that they were overlooked, for M 5 colonies on blood-agar plates at partial tension are a vivid green—as are those of typhoid, paratyphoid B. and B. enteritidis.

Antigens from all strains tested were prepared at one time and kept under the same conditions. Cultures were washed and suspended in 0.9% salt solution, killed at 65 C. for 30 minutes, freed of clumps and preserved with 0.5% phenol.

Agglutinating serum for B. influenzae and the Koch-Weeks bacillus were prepared by inoculating rabbits with living cultures. In the case of M 5 a dead antigen was necessary. The serums had a rather low titer, about 1:800.

TABLE 1

AGGLUTINATION AND ABSORPTION REACTIONS

Designation of Strains	Mother Antiserum		Koch-Weeks Antiserum	
	Simple Agglutination	Homologous Agglutinins Absorbed	Simple Agglutination	Homologous Agglutinins Absorbed
1. Mother	+	+	—	
2. B.	+	+	—	
3. H.	+	P	+	P
4. S.	+	+	—	
5. M.	+	+	—	
6. S.	—	..	—	
7. C.	+	P	—	
8. A.	—	..	—	
9. Sum.	—	..	—	
10. K.	—	..	—	
11. Leo	—	..	—	
12. Koch-Weeks	—	..	+	+

+ means positive agglutination.
— means no agglutination.
P means homologous agglutinins.
P means homologous agglutinins only partially absorbed. When simple agglutination tests were negative absorption tests were not made.

As shown in table 1, four of the strains from the family S., mother, B., S. and M., and strain C from a healthy individual, are probably identical. One other of the family strains, H., is somewhat related to this group but is about as closely related to the Koch-Weeks bacillus. This agrees with the work of others in showing that there are distinct serologic groups among the influenza bacilli and that some strains are closely related to the Koch-Weeks bacillus.

Blood serum from some of the recovered members of family S., who had harbored B. influenzae, contained no agglutinins for this organism.

ATTEMPTS TO TRANSMIT INFLUENZA TO WHITE MICE AND RATS, GUINEA-PIGS AND RABBITS BY MEANS OF SPRAYED SPUTUM

Sputum, or, when this could not be obtained, material swabbed from the tonsillar area, but generally both, were thoroughly shaken with 0.9% salt solution and sprayed by means of a Vilbiss atomizer within half an hour after collection. The animals were placed in a metal box about 12 x 10 x 6 inches, provided with a glass window and air outlets. The spraying was continued until the chamber was filled with vapor. This was repeated at intervals until 15-20 c c had been sprayed. After from 30-60 minutes the animals were removed from the cage and each series kept in separate cages. These were scalded with hot water before they were used for any given series. The sawdust bedding was not sterilized. Precautions were taken to sterilize the drinking pans in the case of all mouse experiments. The animals were fed on cracked maize and vegetable waste from the hospital kitchen.

It might be noted here that one of us in Cincinnati inoculated sterile milk with influenza sputum (1919) and incubated it at 37, 24 and 15 degrees for from 1-14 days, and fed it to white rats and white mice. Of about 40 animals so fed only 2 mice died, one with pneumococcus septicemia, and one with pneumonia and serous pleuritis due to 4 different bacteria. None of these bacteria, singly or combined, produced infection when fed to other mice in milk or broth cultures.

Table 2 gives the data on these experiments.

Family S., Sputum Spray: Four of 5 mice died infected with a strain of B. enteritidis (type M 5); two of these died of a primary pneumonia due to M 5. By the term primary pneumonia we mean pneumonia without marked involvement of the liver and spleen, which invariably occurs in a general infection, e. g., after feeding.

Of 2 guinea-pigs, 1 died of primary pneumonia due to M 5. One of 2 mice and 1 of 2 guinea-pigs sprayed with extracts of the spleen, liver and lungs of this animal died of general infection with M 5.

Two of 4 rats died of primary pneumonia and the lung cultures yielded no growth, nor were subinoculations of organ extracts fatal to rats.

Partial tension rabbit blood-agar plate cultures, from the sputum used for spraying the animals, were sprayed after 24 hours' growth at 37 degrees. Of 6 mice, 2 guinea-pigs and 1 rabbit, only 3 animals died—2 mice the 31st and

TABLE 2

Influenza-Sputum Spraying Experiment

Date	Material Used to Infect Source	Method of Exposure	Number and Species of Animals Exposed	Number of Animals Fatally Infected	Number of Infected Animals with Pneumonia	Minimum and Maximum Duration of Illness, Days	Percentage of Fatalities	Remarks
2/ 3/20	Sputum S	Spray	5 mice	4 (1 killed)	2 (M1,M2)	19–73	80	All like M5
2/ 3/20	Sputum S	Spray	4 white rats	2	2 (W1,W2)	13	50	Sterile
2/ 3/20	Sputum S	Spray	2 guinea-pigs	2	1 (G.-P. 3)	8–80	100	Like M5
2/22/20	Lung emulsion M1	Intraperitoneal	2 mice	2	0	6–8	100	General infection like M5
2/23/20	Mixed culture M1 plate	Spray	2 mice	2	2 (M5,M6)	8–12	100	M6 like M5
3/ 3/20	Lung emulsion M5	Spray	2 mice	1	1	12	50	Like M5
2/25/20	Organ emulsion M2	Spray	2 mice	0	0	..	0	
2/16/20	Organ emulsion W1, W2	Intraperitoneal	2 white rats	0	0	..	0	
4/23/20	Organ emulsion G.-P. 3	Spray	2 mice	1	0	40	50	
3/23/20	Organ emulsion G.-P. 3	Spray	2 guinea-pigs	1	0	30	50	General infection like M5
2/ 4/20	Sputum cultures case S	Spray	6 mice	2	2	31–45	33	No growth obtained from any tissue
2/ 4/20	Sputum cultures case S	Spray	2 guinea-pigs	0	0	..	0	Killed and found normal 5/2/20
2/ 4/20	Sputum cultures case S	Spray	2 white rats	0	0	..	0	
2/ 4/20	Sputum cultures case S	Spray	1 rabbit	1	1 (R1)	8	100	G + coccus present, lung, large numbers
2/16/20	Culture R1	Intravenously, intraperitoneal	2 rabbits	2	2	34–40	100	Infecting agent not R1
3/ 2/20	Sputum R and S	Spray	2 mice	2	1	33–45	100	Like M5
2/ 2/20	Sputum R and S	Spray	1 guinea-pig	1	1	26	100	Like M5
3/ 3/20	Sputum B and B	Spray	8 mice	4	1	30–38	50	Like M5, one sterile
4/ 4/20	Lung emulsion B and B 30-day mouse	Intraperitoneal	2 mice	1	1	50	50	Like M5
4/ 7/20	Lung emulsion B and B 38 day mouse	Intraperitoneal	1 mouse	1	0	3	100	Like M5
3/ 5/20	Sputum B and B	Spray	6 mice	1	0	52	...	Like M5 from lung
3/ 5/20	Sputum T	Spray	6 mice	2	0	72–84	33	Like M5

45th day were sterile bacteriologically, and 1 rabbit of primary pneumonia, apparently due to a gram-positive coccus, which, however, did not reappear in the 2 subinoculated rabbits.

R. and S. Sputum Spray.—The 2 mice sprayed died of infection with M 5, one of primary pneumonia. The guinea-pig died of primary pneumonia due to M 5.

B. and B. Sputum of 3/3.—Of 8 mice, 4 died, but only 1 of these had pneumonia due to M 5. Two had general infection due to M 5.

B. and B. Sputum of 3/5.—Of 6 mice sprayed 1 died on the 52nd day of general infection with M 5. The rest which were killed 3 months later, were found to be normal and did not harbor M 5.

Sputum T.—Of 6 mice sprayed, 2 died on the 72nd and 84th days thereafter; 1 had a general infection with M 5. The remaining 4 were killed 3 months later and found to be normal.

Summary.—The animals sprayed with influenza sputum comprised 33 white mice, of which 4 died of primary pneumonia and 9 of a general infection with M 5. Of this same lot of mice, 6 were sprayed with culture material and of these 2, which died, were sterile on bacteriologic examination; 2 were killed and cultured 2 months later and found to be uninfected; and 2 were used for another experiment in which they survived for a month.

Of 5 guinea-pigs, 2 died of primary pneumonia; the lungs of these animals contained numerous B. enteritidis which were culturally and serologically identical (agglutination and absorption) with M 5.

Of 6 white rats similarly exposed, 2 died of a primary pneumonia due to an unrecognized cause and not transmissible to rats by intraperitoneal inoculation.

The work of Krumwiede, Valentine and Kohn [1] shows that these animals may develop spontaneous infection with members of the paratyphoid-enteritidis group. We did not encounter a single death among our unused stock due to such bacteria, nor were we able to isolate such bacteria from the intestinal tract, liver, spleen and lungs of 6 normal mice. However, the experiments detailed in table 7 show that a certain number of mice, which are intoxicated by killed cultures of M 5 or by the sterile Berkefeld filtrate of broth cultures, develop a secondary infection with M 5. In such endogenous infections, following intoxication, pneumonia occurred only twice in 40 animals.

Furthermore, of 29 mice sprayed with a virulent culture of the pneumococcus, only 1 died of infection with M 5, and this mouse had

[1] Jour. Med. Res., 1919. 39, p. 449.

received a previous dose of M 5 toxin. On the other hand, as shown in table 5, mice exposed to sprayed cultures of M 5 almost invariably died of a primary pneumonia.

In the light of these data one is tempted to believe that the animals developing infection with M 5 were injured in some way by something in the influenza sputum. Nevertheless, the possibility of purely spontaneous infection exists and the question can only be settled by further work with more adequate controls, i. e., an equal number of animals from each lot used for an experiment should have been kept under identical conditions as controls.

Inoculation of Other Animals

From one of the typical cases, "R," 20 c c of blood was obtained. This was used to inoculate a series of animals not generally used in laboratory experiments with the hope that a susceptible animal might be encountered. These were a pig. 8 weeks old, weighing about 100 pounds, a ferret, an opossum, a salamander, and a black headed nun. None showed any abnormal symptoms during 3 months' observation.

The Cultural and Agglutinative Relationship of M 5 (Tables 3 and 4)

Since all the enteritidis-like organisms isolated from the mice and guinea-pigs exposed to sputum sprays corresponded in their agglutination, absorption and cultural characteristics, we used M 5 alone for the comparative study. Unfortunately only two antiserums, M 5, with a titer of 1:800, and paratyphoid B., with a titer of 1:10,000, were available. Table 3 shows that M 5 is entirely distinct from paratyphoid B., but that it is indistinguishable by this test alone from Danysz virus and from B. enteritidis. However, the cultural results (table 4) show that Danysz virus agrees with paratyphoid B. in its failure to ferment xylose, while M 5 agrees with B. enteritidis in the fermentation of this substance. This divergence was brought out by Krumweide et al.[2] We have been helped also in this study by reference to the work of Jordan[3] and preceding articles cited here and that of Winslow, Kligler and Rothberg.[4] The Danysz virus and paratyphoid B. were from the U. S. Hygienic Laboratory and the B. enteritidis was of the Gaertner type and came from Prof. E. O. Jordan in 1901.

Five cultures of B. enteritidis-like organisms isolated from the stools of influenza patients by Sherwood, Downs, and McNaught[5] were sent by Dr. Sherwood. None of these agglutinated with M 5 antiserum.

Experiments Showing That Broth Cultures of M 5 Contain a Soluble Toxin with Which an Antitoxin May Be Produced

Plain, maltose and dextrose beef infusion broths were tried. It was found that 0.1% dextrose broth (+0.5) yielded the most potent toxin. After incubation at 37 C. for 4-5 days the culture was filtered through a Berkefeld N. The filtrate would kill mice in 12-18 hours when 0.05-0.1 c c was injected intraperitoneally. Seventy mice were used in establishing the nature and

[2] Jour. Med. Res., 1919, 39, p. 449.
[3] Jour. Infect. Dis., 1920, 26, p. 427.
[4] Jour. Bacteriol., 1919, 4, p. 429.
[5] Jour. Infect. Dis., 1920, 26, p. 16.

potency of this toxin. Mice dying of intoxication showed marked injection of the subcutis and congestion of the lungs. Often the pulmonary congestion bordered on consolidation. The lungs often showed numerous capillary hemorrhages. Other organs and tissues appeared normal to the eye.

We found that about 2 c c was the M L D, on intravenous inoculation, for a rabbit weighing 1,800 gm. By inoculation with sublethal doses and gradually increasing the amount at 2-day intervals, over a period of 3 weeks, a

TABLE 3

SIMPLE AGGLUTINATION OF CERTAIN STRAINS BY B. PARATYPH. B. AND M 5 ANTISERUM AND THE ABSORPTION OF HOMOLOGOUS AGGLUTININS FROM THE SAME

Designation of Strains	Para B. Antiserum		M5 Antiserum	
	Simple Agglutination	Homologous Agglutinins Absorbed	Simple Agglutination	Homologous Agglutinins Absorbed
M-5	—		+	+
Para B	+	+	+	—
Enteritidis	—		+	+
Danysz virus	—		+	+

TABLE 4

THE MORPHOLOGIC AND CULTURAL CHARACTERISTICS OF M 5 AND OF REPRESENTATIVE ORGANISMS SELECTED FROM THE SAME GROUP

	Gram Stain	Motility	Litmus Milk: Initial Acid	Litmus Milk: Final Strongly Alkaline	Indol	H_2S Produced	Gelatin Liquified	Voges Proskauer	Endo's Acid	Green Colonies Partial Tension Blood Agar Plates	Dextrose	Maltose	Galactose	Saccharose	Levulose	Lactose	Mannite	Arabinose	Dulcite	Erythrite	Isodulcite	Mannose	Raffinose	Salacin	Xylose
M5	—	+	+	+	—	+	—	—	—	+	ag*	ag	ag	—	ag	—	ag	ag	ag	—	ag	ag	—	—	ag
Danysz virus	—	+	+	+	—	+	—	—	—	—	ag	ag	ag	—	ag	—	ag	ag	ag	—	ag	ag	—	—	—
B. Para. B.	—	+	+	+	—	+	—	—	—	+	ag	ag	ag	—	ag	—	ag	ag	ag	—	ag	ag	—	—	—
Enteritidis	—	+	+	+	—	+	—	—	—	+	ag	ag	ag	—	ag	—	ag	ag	ag	—	ag	ag	—	—	ag

ag = acid and gas.

rabbit could tolerate 5 c c of a freshly prepared toxin. Eight days after the last dose of toxin the serum of this rabbit would protect mice when mixed with 2 M L D of the toxin and at once inoculated intraperitoneally.

In the experiments summarized in table 7 the vaccine was prepared by suspending an agar culture in 0.9% salt solution, heating at 65 C. for 60 minutes and preserving with 0.5% carbolic acid. The density was somewhat greater than that of a 24-hour broth culture of B. typhosus. The dosage was approximately 2 minims for each inoculation. Several tests of the vaccine before and after its use showed that the bacilli were dead.

TABLE 5

EXPERIMENTS SHOWING THAT WHEN ANIMALS ARE EXPOSED TO THE SPRAY OF M 5 CULTURES A LARGE PERCENTAGE DELEVELOP PNEUMONIA, AND THAT SIMILAR RESULTS FOLLOW THE SPRAY OF DANYSZ VIRUS; WHILE A SPRAY OF B. INFLUENZAE ISOLATED 48 HOURS PREVIOUSLY FROM AN INFLUENZA LUNG WAS WITHOUT EFFECT

Date	Animals	Number of Deaths	Percentage of Dead Animals with Primary Pneumonia	Duration of Illness in Days	Remarks
3/12/20	4 mice	4	100	4-20	
3/17/20	10 mice	8	100	4-15	
3/30/20	4 mice	4	75	1-10	Sublethal dose M5 toxin given few hours before spray. One toxin death
3/25/20	5 mice	4	100	4-12	
4/8/20	1 mouse	1	0	16	
5/11/20	2 guinea-pigs	2	100	11-17	
5/5/20	6 mice	5	100	12-20	Danysz virus spray
	4 mice	0	...	.....	B. influenzae

TABLE 6

EXPERIMENTS SHOWING FAILURE TO IMMUNIZE AGAINST M 5 CULTURE SPRAY BY PREVIOUS INOCULATIONS OF SUBLETHAL DOSES OF M 5 SOLUBLE TOXIN

Date	Number of Mice	Number of Deaths	Duration of Illness in Days	Remarks
4/6/20	4	3	11-18	One dose soluble toxin 6 days before spray
4/6/20	5	4	12-44	Four doses soluble toxin at 3 day intervals. Last dose 5 days before exposure to spray
4/6/20	4	1	14	Controls without toxin

TABLE 7

EXPERIMENTS SHOWING FAILURE TO IMMUNIZE AGAINST M 5 SPRAY BY PREVIOUS SUBCUTANEOUS INOCULATION WITH A KILLED CULTURE

Date	Number of Mice	Number of Deaths	Percentage of Dead Animals with Primary Pneumonia	Duration of Illness in Days	Remarks
5/7/20	4	4	100	12-23	One dose 10 days before exposure to spray
5/7/20	4	3	33*	1*- 8	Two doses at 4 day interval. Last dose 10 days before spray
5/7/20	4	3	66*	8-14	Three doses at 4 day interval. Last dose 10 days before spray
5/7/20	5	5	80	6-14	Controls not vaccinated

* Two mice died of general infection with M 5 before the time for exposure to the spray.

Experiments Showing That Primary Pneumonia Is Not Produced in Mice When M 5 Is Inoculated by Other Means Than Spray

When the portal of entry was through the conjunctiva, the buccal and gastro-intestinal mucosa, the subcutis or peritoneal cavity, general infection followed. In two cases there was a secondary pneumonia. In such cases the liver and spleen were greatly enlarged and full of whitish necrotic or proliferative areas. These organic lesions are not present in mice dying of inhalation pneumonia. Placing the culture on the nasal mucosa was without effect.

The mice fed M 5 were kept without food and water for from 1-2 days and then each one was watched while it lapped up the drops of a 24-hour broth culture. Those fed heavily ate bread soaked with the broth culture.

TABLE 8

Experiments Showing that Primary Pneumonia is not Produced in Mice when M 5 is Inoculated by Other Means than Spray

Date	Method of Inoculation	Number of Mice	Number of Deaths	Percentage with Pneumonia	Duration of Illness in Days	Remarks
3/18/20	Fed heavily	4	3	0	6-14	General infection
3/31/20	Intraperitoneal	4	4	0	2-4	General infection
4/20/20	Fed 1 drop	4	1	0	8	General infection
4/20/20	Fed 5 drops	4	3	0	10-18	General infection
4/20/20	Fed heavily	2	2	50	8-9	General infection, one with secondary pneumonia
4/20/20	Fed heavily	2	2	0	12-17	General infection
5/11/20	Subcutaneous	3	3	0	2-5	General infection
5/11/20	Ocular conjunctiva	3	2	50	9-19	General infection, one with secondary pneumonia
5/11/20	Anterior nares	4	0			

Experiments Showing That Sprayed Bacteria Are Actually Inhaled Into the Deepest Parts of the Lungs

(a) Four mice and one guinea-pig were sprayed with a broth culture of M 5, and chloroformed within 30 minutes from the beginning of the experiment. They were wet with alcohol and immersed in 1 : 1,000 bichlorid of mercury for 5 minutes. Then they were dissected with aseptic precautions and from each animal 4-6 small pieces of the lungs (1-3 mm. in diameter) were snipped off with sterile scissors and planted in broth. In every instance all the pieces yielded growth of M 5 within 24 hours. Many of the pieces of lung represented the extreme distal portions of the anterior and posterior lobes. Five normal mice controls were treated in the same way. All cultures from these remained sterile during 72 hours' observation.

(b) Experiment (a) was duplicated except that a very virulent pneumococcus (type 1) was used and the tissue was planted in glucose rabbit blood broth. The 4 mice were killed and cultured as in experiment (a) at 2, 4, 8 and 18 hour intervals after the spray. In every instance the cultures showed that pneumococci were present in the deepest parts of the lungs. Cultures from one control mouse yielded no growth in the same medium.

Attempts to Produce Pneumonia in Mice by Spraying Pneumococci

Having shown that sprayed bacteria reach the deepest alveoli, or capillary bronchi, of the lungs and that pneumococci planted in this way survive in the lungs of mice for at least 18 hours, the maximum period tested, we made the following experiments with a type 1 pneumococcus. This culture had been kept highly virulent for mice at the U. S. Hygienic Laboratory.

Four mice were sprayed with the growth from 4 blood agar slants suspended in broth. They were exposed for 30 minutes. Two died of primary lobar pneumonia in 14 days. No bacteria could be found in the purulent exudate and all cultures remained sterile. The remaining 2 were killed 6 weeks later. They appeared normal and cultures from the lungs remained sterile.

Ten mice were kept at 8 C. for 4 hours and then sprayed. They felt warm on removal from the icebox. Two were killed and cultured shortly after spraying. Pneumococci grew out of all pieces of their lungs, including the most distal portions. At the end of 4 weeks the remaining 8 mice were chloroformed and cultured with negative results.

Since M 5 soluble toxin injures the lungs of mice, 6 mice which had survived sublethal doses of this toxin given 10 days before were sprayed. They were killed 6 weeks later and found normal. Nor did they harbor pneumococci.

Four mice were given sublethal doses of M 5 toxin and sprayed with pneumococci at once. Six weeks later one of these mice died of pneumonia caused by M 5; no pneumococci could be found. The remaining 3 were killed and cultured 8 weeks later. They were normal and the cultures remained sterile.

Three mice were sprayed with the pneumococcus and then in an attempt to give them an acidosis they were kept under ether for one hour. One of these was further chilled in ice water for 10 minutes. They survived and yielded no growths 4 weeks later.

Two mice were sprayed with a broth suspension of bloody sputum from a case of pneumococcus lobar pneumonia. They remained well during 6 weeks' observation.

Since none of the 29 mice became infected after inhaling virulent pneumococci into their lungs, one may conclude that some predisposing factor must precede or accompany such an implantation of bacteria. While we owe the whole idea of droplet infection to Flügge and his pupils and confess that we have relied on the review of their work by Goetschlich,[6] we are not aware of the fact if these workers demonstrated that bacteria are to be recovered from the deepest portions of the lungs of sprayed animals. Our attention was drawn to this by Rogers,[7] who showed that tubercle bacilli could be recovered from the lungs of guinea-pigs immediately after spraying them with tuberculous sputum, and that such protected and sprayed animals develop true primary pulmonary tuberculosis.

We are familiar with the work of Dürck [8] who, by means of intratracheal insufflation was unable to infect the lungs of rabbits with

[6] Handbuch d. path. Mikroorg., Kolle and Wassermann, 1914, vol. 2.

[7] Amer. Rev. Tuberc., 1919, 3, p. 238, and 1920, 3, p. 750.

[8] Deut. Arch. f. klin. Med., 1897, 58, p. 368.

freshly isolated cultures of pneumococcus, streptococcus pyogenes, and staphylococcus aureus unless at the same time, or before or after, injurious dust particles, pumice, or "Thomasphosphatmehl," were also blown into the lungs. This sterile dust alone produced pneumonia while sterile street dust did not. He also describes the production of typical pneumonia in rabbits, with secondary invasion of the pneumonic areas by B. coli, sarcinae, or Friedländer's bacillus, by keeping them at 37-41 C. for 16 to 36 hours and then in ice water for 2-7 minutes.

However, these experiments and those made by the method of intrabronchial insufflation, which was introduced by Lamar and Meltzer and used by many others, do not appeal to us as representing what must take place under natural conditions. Bacteria can be inhaled into the deepest parts of the lungs and if they are capable of multiplying there they will produce pneumonia, as in the case of M 5. The fact that virulent pneumococci do not multiply when planted in the lungs of mice by air currents is an interesting fact and deserves further investigation.

SUMMARY AND CONCLUSIONS

When white mice, white rats and guinea-pigs were exposed to finely divided influenza sputum sprays some died of a primary pneumonia, others of a general infection due to a strain of B. enteritidis (type M 5). Since the work of others has shown that these animals may die of spontaneous infection with members of the paratyphoid-enteritidis group we cannot say that these infections were necessarily the sequel to the spray. However, as primary pneumonia could not be produced in mice when M 5 was inoculated through the buccal or gastro-intestinal mucosa, the conjunctiva, subcutis or peritoneal cavity, but only when sprayed, it seems to us likely that something in the sputum sprays produced a change in the pulmonary tissues favoring such secondary localization.

Broth cultures of M 5 contain a soluble toxin which produces marked congestion of the subcutaneous and pulmonary tissues of white mice. This toxin gives rise to an antitoxin when injected into rabbits. Previous inoculation with the toxin did not produce immunity to the development of primary pneumonia by sprayed cultures, nor were we able to immunize against the spray of M 5 cultures by previous subcutaneous inoculations with a dead culture.

The intoxication of mice with the soluble toxin or with killed cultures of M 5 apparently led to infection with M 5 in a small percentage of the used mice. We were not able to find this bacterium in normal mice, nor did spraying mice with virulent pneumococci make it show itself as a secondary invader.

Experiments show that M 5 and virulent pneumococci are inhaled by mice into the deepest alveoli or capillary bronchi of the lungs, and that primary pneumonia follows in the case of M 5 which is capable of growing and producing its toxin there, whereas, the virulent pneumococci gradually disappear.

THE FATE OF KILLED NONHEMOLYTIC STREPTOCOCCI INJECTED INTO THE BLOOD, AND THE RESULTING CELLULAR CHANGES

WITH PLATE 1

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I. THE GENERAL RESULTS

In connection with work on the fate of India ink and of tubercle bacilli when introduced into the blood, the results of which are yet to be published, I have studied also the fate of nonhemolytic streptococci and the resulting cellular changes. On account of the difference in the reactions that may follow the introduction of bacteria into the blood, depending on whether the bacteria are virulent or nonpathogenic, it is impossible to determine beforehand what the fate of a given bacterium may be in a certain animal on the basis of what we know at present about these questions.

Wissokowitsch,[1] as early as 1886, showed that both pathogenic and nonpathogenic bacteria introduced into the blood either decrease greatly or disappear wholly from the circulation in a short time, and that the bacteria are stored in the liver, spleen and marrow, partly in the endothelial cells, partly in the capillaries. Others (Werigo,[2] Arima,[3] Römer,[4] Filatow[5]) also found that these organs, especially the liver and spleen, were of great importance in ridding the body of bacteria. Kyes[6] ascribes the immunity of the pigeon to the pneumococcus to the phagocytic action of the endothelial cells of the liver and spleen rather than to the action of the fluids of the body or to its high temperature. Bartlett and Ozaki[7] and Hopkins and Parker[8] observed that certain bacteria introduced into the blood accumulate in the vessels of the lung rather than of the other organs. The work mentioned, as well as that of others, will be discussed more in detail later.

I selected a nonhemolytic streptococcus because it is easy to recognize the cocci in the tissues and the blood, and because when thoroughly broken up into its simple form, it does not tend to form

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[1] Ztschr. f. Hyg. u. Infectionskh., 1886, 1, p. 2.
[2] Ann. d. l'Inst. Pasteur, 1894, 8, p. 1.
[3] Mitteilungen d. Med. Gesellsch. zu Osaka, 1910, 9, p. 962; Arch. f. Hygiene, 1911, 73, p. 265.
[4] Arch. f. Augenheilkunde, 1906, 55, p. 313.
[5] Arch. f. Augenheilkunde, 1911-12, 70, p. 185.
[6] Jour. Infect. Dis., 1917, 18, p. 277.
[7] Jour. Med. Res., 1917, 35, p. 466.
[8] Jour. Exp. Med., 1918, 27, p. 1.

aggregates. Killed cocci were used in order to make the process as simple as possible by eliminating growth and the formation of toxic substances.

A 24-hour growth of the streptococcus in 180 c c of ascites dextrose broth was collected by centrifugation, washed three times with salt solution, and suspended in 50 c c of this solution. This suspension was shaken violently for several hours when 40% formaldehyd was added in the ratio of 1%, the shaking repeated and the suspension left to stand for at least 18 hours. The cocci were collected once more and washed several times, then suspended in 2 c c of salt solution for every 10 c c of the original culture, and shaken again. The suspension was then left standing and the upper part removed from time to time. In this way a suspension of single cocci was obtained with but few chains of 3 members. About 2.5 c c were injected into the jugular vein of guinea-pigs weighing about 300 gm. In a few cases the injection was made into the left ventricle of the heart.

After the injection the animals were killed, one immediately and then at intervals of 5, 10 and 30 minutes, and in 1, 2, 6, 12, 48, 72 and 96 hours.

The organs and tissues were removed immediately after death and placed in Zenker's fluid. The marrow, which was the last to be removed, sometimes did not reach the fluid until after 20 to 30 minutes. Five micron sections were made and stained with Delafield's hematoxylin and eosin and a modification of the Gram-Weigert stain, using the following method:

1. Paraffin removed in xylol.
2. Absolute alcohol, then 95% alcohol, then in 1% solution of iodin in 95% alcohol for about 10 minutes, and then in 1% sodium carbonate.
3. Running water for 10 minutes followed by distilled water.
4. Hematoxylin 8 to 10 minutes.
5. Destained in 1% hydrochloric acid in 70% alcohol until blue color almost vanished.
6. Running water for 30 minutes, then in 0.2% aqueous solution of eosin for one minute, followed by running water for 10 minutes or more.
7. Three per cent. carbol gentian violet 30 minutes in incubator, then washed with 2% carbolic acid solution in water and placed in Lugol solution for 1 to 2 minutes.
8. Washed with water, blotted with filter paper, cleared with anilin oil and xylol equal parts followed by several changes of xylol; mounting in xylol balsam.

The method is a modification of Czaplewski,[9] in which phenol solution is used instead of anilin oil water.

Special attention was given to the relation between the cocci and the cells, particularly the endothelial cells, in all the organs and tissues, but in spite of the great care and extent of examination the results seem relatively meager. Briefly stated, the cocci were found in cells for only a short time after the injection, and almost exclusively in the endothelial cells of the liver, the spleen, and in the so-called spleen cells, and the leukocytes. The polymorphonuclear pseudo-eosinophil

[9] Schmorl, Untersuchungensmethoden, 1914, p. 311.

leukocytes seemed to be very active so that within 5-10 minutes after the injection practically all the cocci seemed to have been taken up by the leukocytes, particularly those in the capillaries of the lungs, but even immediately after injection cocci were found in the endothelial cells of the liver and spleen; it is possible that these cells were phagocytically active even after the death of the animal. Practically no phagocytosis was observed on the part of the endothelial cells of the other organs and the tissues, including the brain and spinal cord as well as the large vessels. In the lungs, suprarenals, cortex of the kidney and the glomeruli suggestions of phagocytosis were seen sometimes, and in that case only one or two cocci were found in each cell. It was rather surprising that marrow, the endothelial cells of which take up India ink granules actively as well as particles of blood pigment, showed no distinct phagocytic action for the cocci, only a few of which surprisingly enough were ever found in the marrow. As stated, the leukocytes in the blood stream took up cocci quickly and in large numbers, the large mononuclears in lesser degree, and in films made before death leukocytes with cocci in them could be found as long as 3 hours after the injection, but only rarely after that time. Free cocci could be found in the blood for only about 30 minutes after the injection, mostly entangled in masses of platelets. Within the cells the cocci seemed to lose rapidly their tingibility and to become smaller, finally forming small red granules before complete disappearance, which usually took place within 72 to 96 hours. The liver seemed the most active in the removal of the cocci, the spleen being next in order. Within a short time after injection definite cellular reactions developed in these organs as well as in the marrow, the lungs, the kidneys and the suprarenals. The bone marrow showed great increase in the number of polymorphonuclear leukocytes as soon as 10 minutes after the injection, even though hardly any cocci were present; later these leukocytes diminished in number while transitional forms seemed to increase, being like the myelocytes rather larger than normally, the nuclei coarser. The increase in leukocytes in the marrow reached its maximum in about 30 minutes after the injection to fall again after about 3 hours followed by a second wave of increase in about 3 hours more. The megakaryocytes seemed to undergo degeneration with pyknosis, some showing mitosis. The endothelial cells of the liver would vary in size and staining; a specially large type with a round or oval nucleus and a large amount of cytoplasm would arise

and project into the lumen of the capillary as if about to fall off. Such cells were actively phagocytic. In the spleen a great number of plasma cells appeared soon after the injection, arising from lymphocytes, the nuclei of the two kinds of cells appearing alike. There also appeared in the spleen a degenerate type of cell from pycnosis or karyorrhexis of lymphocytes, which was found in the pulp as well as in the malpighian bodies. The endothelial and reticular cells seemed large and swollen. In the kidney and suprarenals the endothelial cells seemed large also, with increased chromatin, and in some cases there was interstitial proliferation. In the lungs the endothelial cells seemed to react in the same way and to give rise to some of the mononuclear cells in the lung. Similar changes as in the spleen were found in lymph nodes in various parts. In the submucosa of the stomach, and especially of the intestines as well as in the uterus, the number of plasma cells appeared to increase greatly after the injection.

Lungs.—Here the bacteria accumulate first in the course of their distribution after being injected into the jugular vein. Almost immediately innumerable cocci were found in the lungs, often accumulating in the capillaries, sometimes to a marked degree, the capillaries dilating from local toxic action. Even at this time the cocci were mostly found within polymorphonuclear leukocytes, a few being seen also in mononuclear cells and in endothelial cells. After 10 minutes the number of polymorphonuclears seemed to have increased and almost all the cocci had been taken up. In the course of the next 20 minutes or so the leukocytes became scattered in the circulation and the number of cocci seen smaller; after 2-3 hours the bacteria became few, and at the end of 24 hours the condition seemed quite normal.

Werigo was the first to observe that bacteria introduced into the blood stream accumulate first in the lungs whence they are transported elsewhere by leukocytes. He injected rabbits with anthrax bacilli. He says that it is evident that in these early stages after injection the lungs represent a source of infection of the circulating blood by the bacteria. Arima found by cultural methods that after injection of colon and typhoid bacilli and of staphylococci the number of organisms in the lungs decrease rapidly in the course of the first 3 hours. He thinks that the diminution of bacteria in the lung is due to the bactericidal action of the blood on one hand and to transportation elsewhere by the blood on the other hand. Bartlett and Ozaki, who injected staphylococci into the left ventricle in dogs, also found by microscopic study that the number of cocci diminished greatly during the first 20 minutes after the injection. At first the cocci were free in the capillaries, but in the course of 10 minutes or so they were taken up by leukocytes, being either destroyed quickly or carried to other parts, principally the liver and spleen. Hopkins and Barker, working with hemolytic streptococci, were impressed with the streptococcidal action of the lung tissue, and the details of their work will be referred to later. All observers seem to agree that bacteria injected into the blood accumulate first in the capillaries of the lung, where they become subject to phagocytosis in a short time so that in 30 minutes or so most of them are removed, the lungs being apparently quite free in about 3 hours, until, according to Werigo, bacteria may

begin to accumulate again in the lungs. In the experiments just cited living organisms were used, and it might be assumed that different results would be obtained with dead organisms, but my results agree very well with those of the aforesaid experiments. In my experiments endothelial and mononuclear cells took more part in the action than in Werigo's experiments with anthrax bacilli. Bartlett and Ozaki, as well as Hopkins and Parker, noted similar action on the part of these cells. Hopkins and Parker concluded that streptococci taken up by the cells in the lung were killed within 5-8 hours although they remained visible in the sections after a number of days, the destruction being due to the action of living cells as extracts of the lung had no destructive effect. In my experiments, however, there seems little doubt but that the bacteria disappeared from the lungs principally as the result of transport by leukocytes to other places and also as the result of digestion within the phagocytes. I would emphasize especially the fact that the cocci on injection accumulate in the lung capillaries even when the injection of carefully made suspensions was done slowly and gradually. Bull[10] observed that certain bacteria, when injected intravenously into rabbits, would be agglutinated while others would not unless the animals were previously injected with a corresponding antiserum. He believes that the agglutination in the blood is favorable to the elimination of the organisms as the leukocytes can take up large numbers of bacteria more readily. In my experiments, the cocci seemed to accumulate loosely without being first agglutinated, and it seemed to me that this resulted in a measure from the failure of the suspension to mix well with the blood, the bacteria gathering first in the lungs and leading to the accumulation of leukocytes, some of course being carried directly through the lung into the blood. As the bacteria form collections in the blood in the lung the distribution later to other parts, especially the liver, is irregular and many phagocytic endothelial cells may be loaded with bacteria while others have few or none. There may be differences in phagocytic activities also, but that would hardly account for the irregular distribution.

When the same quantity of bacterial suspension that was injected into the jugular vein was introduced slowly into the portal vein and the animal killed five minutes later, the appearances in the liver and the lung were quite the reverse of the appearances after jugular injection. Not only did more endothelial cells in the liver contain cocci, but many polymorphonuclear leukocytes appeared also in the vessels of the liver, while in the lung there were hardly any changes except that some endothelial and mononuclear cells containing a few cocci were present.

Bartlett and Ozaki found that bacteria injected into the left ventricle gathered in large numbers in the lung. I made careful comparison in 3 guinea-pigs, in 2 of which the coccal suspension was injected into the left ventricle, the animals being killed 5 and 10 minutes later, while a third animal received injections into the jugular vein and killed after 10 minutes. There were few cocci in the lungs of the animals that had received injections into the left ventricle as compared with the number in the animal receiving injections into the jugular vein; in the former animals the liver contained the largest number of cocci.

It may be concluded that the bacteria accumulate in the lungs after intravenous injection largely as the result of mechanical influences, notably the difficulty of dispersing the bacteria in the blood, and the

[10] Jour. Exper. Med., 1915, 22, p. 457, 475, 484.

small size of the capillaries, to which must be added the secondary accumulation of leukocytes. As shown in my work with India ink, the granules seem to adhere first to the surface of the phagocytes, but the lungs do not contain as many active phagocytic cells as the liver and spleen. This is another reason why the accumulation of bacteria in the lungs is ascribed largely to influences of a mechanical nature.

Table 1 shows the distribution of polymorphonuclear leukocytes in the organs of four normal guinea-pigs, the number of phagocytic endothelial cells and leukocytes in relation to the total number of such cells at various periods after the injection as well as the total number of phagocytic cells and of leukocytes in all the organs examined. While it might have been more desirable to count the cocci, this seemed to be impracticable because of the formation of lumps and the development of changes so that one coccus could not be distinguished from another, and on this account the average number of cells containing cocci was counted. The figures in the table give the average number of cells in each microscopic field as determined by counting 90 fields from at least 3 different sections, all 5 mikrons in thickness, in each case. Ocular 4 and oil immersion objective 1.8 were used. In the liver the interior of the acini, excluding Glisson's capsules and the central vein, was studied. In the spleen the pulp only was examined. In the case of the marrow no attempt was made to classify the cells definitely, most of them being polymorphonuclears. In order to count all the leukocytes in the marrow the fields were divided into 4 areas and the cells enumerated in 1 area multiplied by 4. While the figures are not to be regarded as exactly representing the numbers of phagocytic and other cells, they represent the periodical variations in the different organs far more accurately than statements based on general observations could.

Table 1 shows that in the liver there are more phagocytic endothelial cells after 5 minutes than immediately after the injection; then the number falls to increase again, but after 3 hours the number again falls and 48 hours after the injection almost all phagocytic endothelial cells have disappeared. Phagocytic polymorphonuclear leukocytes increase steadily for about 3 hours after the injection, the total number of leukocytes also increasing; after that the number of phagocytic leukocytes decreases gradually; the total number of leukocytes also begins to fall after the third hour, the normal number being reached in 72-96 hours. It should be noted that the number of phagocytic endothelial cells reached its maximum earlier than the leukocytes and fell off earlier also.

In the case of the lungs, the polymorphonuclears which had accumulated here began to disperse about 30 minutes after the injection and in from 1-3 hours most of them had left the lung, going probably mostly to the liver and the spleen, thus explaining the gradual increase in the number of these cells in these organs up to the third hour. At the same time leukocytes no doubt were attracted to the liver by the accumulation of detritus from cocci and cells. Hence the endothelial cells in the liver receive cocci that are free in the blood and that are liberated from leukocytes and other cells as they degenerate and fall to pieces.

As pointed out, bacteria may be destroyed rapidly in phagocytes. Werigo noted that anthrax bacilli began to disintegrate within phagocytes 7½ minutes after injection. At the same time some endothelial cells, as well as leukocytes, disintegrate during this process, and in the case of endothelial cells younger

TABLE 1

Distribution of Leukocytes and Phagocytic Cells in the Organs After Injection of Killed Streptococci

	Lung	Liver				Spleen				Bone-marrow		Kidney		Adrenal		Muscle	Total No. of Leuko-cytes in All Organs
				Leukocytes				Leukocytes		Leukocytes		Leukocytes		Leukocytes			
	No. of Leuko-cytes	Total No. of Phago-cytic Cells	Phago-cytic Endo-thelial Cells	Phago-cytic	Total No.	Total No. of Phago-cytic Cells	Phago-cytic Endo-thelial Cells	Phago-cytic	Total No.	Phago-cytic	Total No	Phago-cytic	Total No.	Phago-cytic	Total No.	Leuko-cytes	
Normal 1	2.67	.....	.....	.....	0.50	.....	.....	.....	25.2	.....	44.4	.....	0.17	.....	0.22	.....	73.2
Normal 2	1.57	.....	.....	.....	0.55	.....	.....	.....	29.2	.....	81.2	.....	0.19	.....	0.18	.....	112.9
Normal 3	2.80	.....	.....	.....	0.65	.....	.....	.....	36.4	.....	54.0	.....	0.10	.....	.....	.....	93.9
Normal 4	2.17	.....	.....	.....	0.70	.....	.....	.....	31.6	.....	86.6	.....	0.13	.....	0.21	.....	121.9
Average	2.31	.....	.....	.....	0.60	.....	.....	.....	30.6	.....	66.6	.....	0.14	.....	0.20	.....	100.5
40 seconds	9.75	1.62	1.17	0.45	1.33	4.30	2.10	2.20	19.95	1.43	106.2	0.01	0.17	.....	.....	0.23	137.6
80 seconds*	.....	.....	.....	.....	.....	.....	.....	.....	9.50	.....	80.2						
5 minutes	19.15	5.46	5.02	0.44	1.73	2.35	1.25	1.10	4.48	1.03	60.0	0.06	0.12	0.01	0.10	0.08	85.7
5 minutes*	.....	.....	.....	.....	.....	.....	.....	.....	8.55	.....	104.8						
10 minutes	40.10	4.92	4.45	0.47	2.27	5.11	4.72	0.39	7.86	0.64	152.6	0.06	0.21	0.08	0.22	0.15	203.4
30 minutes	18.81	3.96	3.05	0.91	3.17	6.35	5.44	0.91	2.86	0.22	217.0	0.10	0.35	0.23	0.33	0.31	251.8
1 hour	9.85	6.65	5.11	1.54	6.11	7.85	7.19	0.66	6.60	0.30	127.0	0.05	0.47	0.03	0.15	0.35	150.6
3 hours	8.65	4.85	2.88	1.97	13.83	10.83	7.50	3.33	28.42	0.70	73.2	0.05	0.81	0.73	5.80	0.33	126.0
6 hours	3.77	2.61	1.90	0.71	3.17	7.41	5.55	1.86	41.33	1.45	126.2	0.09	0.76	0.30	2.10	0.25	175.8
12 hours	4.13	2.10	1.73	0.37	2.87	6.66	5.00	1.66	55.55	0.30	101.7	0.0	0.83	0.03	0.90	0.20	165.6
24 hours	2.86	1.49	1.42	0.07	1.91	4.66	3.50	1.16	19.95	0.06	93.3	0.02	0.11	0.0	0.33	0.08	118.5
48 hours	2.80	0.79	0.78	0.01	1.12	1.93	1.91	0.02	10.95	0.0	76.9	0.0	0.16	0.01	0.53	0.12	93.6
72 hours	2.61	0.08	0.08	0.0	0.58	.....	.....	0.0	12.75	0.0	60.9	0.0	0.14	0.0	0.06	0.05	74.5
96 hours	2.35	0.0	0.0	0.0	0.51	0.0	0.0	0.0	13.42	0.0	57.3	0.0	0.11	0.0	0.12	.....	73.8

* Suspension of different strain of coccus injected. All other observations were made with same suspensions of same strain.

cells may take up the cocci thus set free. Werigo found a large number of bacilli in the liver 7½ minutes after the injection, the number dropping suddenly after 10 minutes and then more slowly until about the sixteenth hour when an increase began. My figures show two high points, one after the first 5 minutes, and the second one hour after the injection, the only difference from Werigo's results being the second climax which I have ascribed as due to divergence of bacteria from the lungs. Arima also found a diminution in the number of bacteria in the liver after 5 minutes and 1 hour, but his results were based on cultures. Bartlett and Ozaki gave no observation at 5 minutes or so, but their highest figures for the liver were obtained 10 minutes and 1 hour after the injection, there being a definite drop between. These figures were based on direct counting of the bacteria and correspond closely to my results.

The discussion of the endothelial phagocytosis in the liver would be incomplete if we did not consider also the fact that such cells may escape into the blood as noted by Kiyiono[11] and myself in our work with India ink. However, only few phagocytic mononuclear cells were found in the blood in these experiments even when the blood was drawn directly from the right ventricle. This was true particularly at three hours after the injection. In the earlier stages when leukocytes collect in the lung, endothelial cells naturally might accumulate there also and thus account in some measure for the decrease in the number of such cells in the liver. Later on, after the second climax, when practically no phagocytic mononuclear cells could be found in the blood from the right ventricle, the small number of phagocytic endothelial cells in the liver is best explained by rapid intracellular digestion of the cocci. The table shows, however, the presence of phagocytic cells after this period, though in diminishing number, indicating that all cocci are not digested promptly. After 96 hours the cocci had disappeared from the endothelial cells. In Bartlett and Ozaki's experiments and in Kyes' experiments with staphylococcus and pneumococcus, respectively, the cocci were found to have disappeared at the end of 72-96 hours.

Shortly after the injection definite changes could be made out in the endothelial cells. Werigo noted changes 7½ minutes after the injection and grouped the cells into "simples et compliques," the latter having many nuclei, due to the fact that in some cases at least they had taken up leukocytes. In my sections such cells were not commonly found in the liver, being, however, fairly abundant in the spleen. A large number of cells with round or elliptical nuclei and abundant cytoplasm—Werigo's "simples"—sometimes projected into the capillary lumen. Frequently they contained many cocci. In half an hour and then on, the maximum being reached at the third hour, these cells showed marked changes, assuming various shapes and sizes and a deeply stained nucleus. Later the cells began to return to the normal condition and after 72-96 hours only a few changed cells were present. Degenerated cells, endothelial and leukocytic, were seen, sometimes with only faintly stained nuclei or no nuclei, most of them containing many bacteria.

When we consider that the bacteria in the endothelial cells of the liver reach a high point one hour after injection, it seems clear that several generations of cells one after the other are required to destroy all the bacteria even though they were dead when injected. As noted, some cocci appear to be destroyed much more rapidly than others, due possibly to changes in the bacteria themselves, the older bacteria being perhaps destroyed more quickly than the younger.

[11] Vitale Karminspeicherung. Jena, 1914, Nisshin-Igaka, 1914, 4, p. 917.

Werigo states that as leukocytes are taken up by larger phagocytic cells (macrophages) the bacteria are transferred to the latter. In regard to the fate of the leukocytes after the delivery of the bacteria he says that most of such leukocytes appear quite normal, evident signs of destruction being observed only rarely so far as observed in the liver; in the spleen, however, the leukocytes taken up by other cells appear to be destroyed. The impression is made that leukocytes engulfed by other cells in the liver may possibly return again to the blood.

We know that the endothelial cells of the liver and spleen are a place of destruction of red corpuscles. Kyes showed that in the pigeon pneumococci are taken up by these hemophagocytes in the liver and spleen. Whatever the fate of healthy leukocytes that may be taken up may be, there is no question but that the dead and dying leukocytes are treated in the same way as red cells and foreign bodies by phagocytic cells, and in that case bacteria such as streptococci within such leukocytes would be passed over to the larger phagocytic cells in a passive manner.'

Spleen.—The cocci were taken up by endothelial cells and spleen cells, but always in small numbers only, groups or masses being found but rarely. In the earlier stages the malpighian bodies did not contain any cocci; later cocci appeared in the reticulo-endothelial cells, but altogether the phagocytic activity of these cells was less than that of the endothelial cells of the liver. Immediately after the injection and 5 minutes later the phagocytic cells were only few in number, increasing gradually for about 3 hours when the maximum was reached to be followed by a gradual disappearance, which became complete in 72-96 hours. Phagocytic polymorphonuclear leukocytes also increased in number for about 3 hours when a gradual diminution set in. Comparing the number of phagocytic endothelial and pulp cells with the number of phagocytic cells in the liver, it was found that while the number was greater in the spleen, the number of bacteria taken up was much smaller than in the case of the liver. Werigo also found that bacteria were destroyed more slowly in the spleen than in the liver and he is opposed to Metchnikoff's view that the bactericidal power of the spleen is greater than that of the liver. If we consider the volumes of the two organs it becomes quite clear that the liver is the most important organ for the elimination of bacteria in the blood stream, the spleen being next in order.

In the normal guinea-pig polymorphonuclear leukocytes are present in fairly large numbers in the spleen, approximately 30.8 per field on the average. Immediately after infection of cocci the number fell to about one-fourth to one-tenth of the normal average; after about 6 hours and continuing for about 6 hours more the number increased above the normal, dropping again below normal after 48 hours. The only other organ or tissue in which leukocytes are present normally in such large numbers is the marrow, and the reasons for this accumulation cannot be discussed now. The interesting point is that in the spleen the number of leukocytes decreased immediately after the injection while in the liver the number increased. It would seem as though the leukocytes in the spleen were sent to other organs and especially the lungs. The number of leukocytes taken up by other cells in the spleen was not large enough by far

to account for the reduction after injection of streptococci. Such phagocytosis occurs and Werigo traces certain granules found in the macrophagocytes of the spleen to digestion of leukocytes. Mallory[12] and Imamura[13] believe that the granules are rather the remains of lymphocytes. The spleen is an inconvenient place to leave for the leukocytes, on account of the construction of the sinuses and the slowness of the blood current, and there would seem to be a good opportunity for macrophagocytes to take up old and degenerate leukocytes. Add to this, positive chemotactic action and it does not seem difficult to understand that large numbers of leukocytes might be removed from the blood in the spleen during the period between 12-24 hours after injection of cocci, a period when there is no special demand for leukocytes elsewhere, and, as shown in Table 3, no increase of leukocytes in the blood. The question remains whether the leukocytes are destroyed by the activity of the phagocytic cells primarily or due to degenerative processes in the leukocytes. This question cannot be answered definitely, but it seems reasonable that it is principally degenerated cells that are disposed of in this way.

The endothelial and reticular cells began to enlarge 10 minutes after the injection, the nuclei becoming large and the cytoplasm of different cells in places seemingly flowing together so that the distinction between endolethial and reticular cells became more difficult. Mitotic and amitotic cell division increased rapidly and the pulp consequently swelled up. The cells in the malpighian bodies also became enlarged and proliferated, the boundaries of the bodies becoming indistinct. The nuclei of lymphocytes seemed to enlarge, being round or roundish and smooth and the germinal centers consequently increased. Under a low power the field seemed to be composed principally of pulp. After 24 hours and later these reactions subsided gradually and at 72 hours and on to 96 normal conditions as a rule became reestablished. In the course of these changes two special cells made their appearance—plasma cells and a degenerate form to be described more fully later.

Bone Marrow.—There were fewer cocci here than in the liver and spleen, and almost all of them were in polymorphonuclear leukocytes. Singularly enough none were found in the endothelial cells of the marrow which take up other foreign bodies actively. The number of cocci was high immediately after the infection and 5 minutes after; then it decreased gradually up to 30 minutes after the injection when an increase set in which reached its maximum at 6 hours followed by diminution so that at 48 hours practically no cocci could be found. The first stage reached its maximum 5 minutes after the injection and was succeeded by a gradual diminution during the next 30 minutes that may be regarded as the expression of the phagocytic activity of polymorphonuclear leukocytes; it corresponded to what was observed in the spleen except that there seemed to be no diminution in the marrow in the total number of leukocytes, due possibly to the rapid production of leukocytes as indicated by rapid increase of their number. It would seem as if phagocytic leukocytes on reaching the marrow and spleen are sent away again promptly so that there is no accumulation of cocci. The marrow strictly speaking is not actively bactericidal as no cocci were found within the cells proper of the marrow, only within the leukocytes.

[12] Jour. Exper. Med. Med., 1898, 3, p. 611.
[13] Nisshin Igaku, 1916, 6, p. 1871

The changes in the marrow were remarkable and occurred early. Hyperemia in the earlier stages was so marked that the capillaries seemed to have been broken down. After 5 minutes intensely stained cells were present and after 10 minutes a great many well lobulated polymorphonuclear cells were present in the parenchyma at the same time as mitosis was increased. After 30 minutes, however, the polymorphonuclears had decreased while transitional forms of a large type had increased with evidence in the form of mitotic figures of active cell proliferation. The nuclei of myelocytes and transitional forms were rich in chromatin and stained well. This general condition continued without any further marked accumulation of polymorphonuclears as observed 10 minutes after injection until after 48 hours when a gradual return to normal set in. As observed by Muir[14] under somewhat similar conditions, during the early stages the megakaryocytes became more distinct and soon passed into a condition of pyknosis with deeply stained shrunken nuclei. While the normal type of this cell prevailed in approximately 92% of the cells during the first 30 minutes after the injection, the pyknotic form increased to 60% during the second hour, the number of pyknotic cells thereafter gradually falling, being still more numerous than immediately after the injection even after 96 hours. Karyorrhectic forms were rarely seen, mitosis occasionally. Often a large nucleolus appeared in young megakaryocytes.

Tables 1 and 2 give details concerning the number of leukocytes at different periods after injection. From the fact that the number of leukocytes in the spleen and of phagocytic leukocytes in both the spleen and marrow decreased soon after injection, I conclude that the marrow in the meantime is sending out leukocytes rapidly and that at the same time new leukocytes arise from myelocytes. As shown in table 2, in 4 guinea-pigs killed within 5 minutes after injection (PI) there was some increase in the number of polymorphonuclears, due as I have assumed to rapid transformation of myelocytes, the change in the nucleus taking place as if by a peristaltic action. The large number of well lobulated polymorphonuclears at 10 minutes and later after the injection (P2), with many transitional forms, supports this assumption. There now ensues a diminution (P3) due seemingly to an exhaustion of the marrow with the appearance of metamyelocytes and myelocytes in the blood and a new type of polymorphonuclear (see part 3). From the point of view of mitosis, there is a slight increase during the first 5 minutes after the injection (P'I), this increase becoming more marked at 30 minutes and 1 hour (P'2) when a drop ensues (P'3). During the period between 12 and 24 hours after the injection there is a second increase in the number of mitosis (P'4) ending in gradual return to the normal.

Returning to the number of leukocytes, we note a second increase of polymorphonuclears in 6-12 hours after the injection (P4), due to the new production of myelocytes. After 24 hours the number decreases as the reaction subsides. This reaction would be largely influenced by the number and virulence of invading bacteria and if maintained as the result of the stimulus of living microbes soon no doubt an exhaustion of the marrow would result.

We may regard the preformed polymorphonuclear leukocytes in the marrow as the first reserves for active service in the blood, the myelocytes as the second reserve, and newly formed immature cells as soldiers of the national army. The stages in the reaction in the marrow with respect to cell formation as indicated by the number of mitoses may be divided as follows (table 2):

P'1, the first ten minutes, a preparatory stage.

P'2, 30 minutes to one hour, first stage of active division.

P'3 3 to 6 hours, preparation for second cell division, continued cell production and beginning of new stage in cell production.

P'4, 12 to 25 hours, second stage of cell division.

P'5, 24 to 96 hours, gradual return to normal state.

Interpreted on the basis of the number of polymorphonuclears in the marrow the reaction seems to fall into the following stages:

P1, first 5 minutes, mobilization of leukocytes and formation of new leukocytes from myelocytes.

TABLE 2

POLYMORPHONUCLEAR LEUKOCYTES AND MITOSIS IN MARROW AFTER INJECTION OF NONHEMOLYTIC STREPTOCOCCI IN GUINEA-PIGS

Normal Guinea-Pigs

	1	2	3	4	Average
Number of polymorphonuclears	44.4	81.2	54.0	86.6	66.6
Number of mitosis	0.24	0.36	0.31	0.35	0.32

Guinea-Pigs Injected with Nonhemolytic Streptococci

Time After Injection	Number of Polymorphonuclears		Number of mitosis	
40 seconds	106.4	P1	0.44	P'1
80 seconds	80.2		0.41	
5 minutes	60.0		0.48	
5 minutes	101.8			
10 minutes	152.6	P2	0.71	
30 minutes	217.0		2.20	P'2
1 hour	127.0	P3	3.11	
3 hours	73.2		1.35	P'3
6 hours	126.2	P4	1.71	
12 hours	101.7		2.20	P'4
24 hours	93.3	P5	2.32	
48 hours	76.9		0.73	P'5
72 hours	60.9		0.52	
96 hours	57.3		0.41	

P2, 10 to 30 minutes, climax of transformation of myelocytes into polymorphonuclears.

P3, 1 to 3 hours, beginning exhaustion of preformed and newly formed leukocytes with production of new leukocytes from the first stage of active cell division (P'2).

P4, 6 to 12 hours, accumulation of leukocytes as the result of cell proliferation (P'2, P'3); during the last two stages myelocytes and metamyelocytes may appear in the blood.

P5, 24 to 96 hours, subsidence of reaction.

The mobilization and production of polymorphonuclears in the marrow after the injection of streptococci proceed rapidly. This point will be discussed further in part 4.

[14] Jour. Pathol. and Bact., 1900 01, 7, p. 161.

II. DEGENERATED CELLS IN THE SPLEEN AND OTHER ORGANS

Besides plasma cells various forms of degenerated cells appear in the spleen. They were seen in small numbers in the earlier stages, but in from 6 to especially 12 hours after the injection they increased rapidly, diminishing again promptly. Such cells occurred free as in macrophagocytes, in the pulp as well as in the follicles, and here more particularly in the germinal centers within reticulo-endothelial cells. The nuclei of these cells varied so much in shape that they can hardly be described, presenting often ring and crescent shapes, and staining from a deep blue to red with many intermediary shades. These cells appeared most abundantly at the time when polymorphonuclear leukocytes were present in large numbers and often subjected to phagocytosis, and some of the degenerate cells may be derived from such leukocytes. Werigo figures bodies like the one in question and regarded them as the remains of polymorphonuclear leukocytes in the interior of phagocytes. Mallory [15] described the same forms in the lymph nodes and other organs in typhoid fever, as well as in the spleen of animals infected with toxic products of the typhoid bacillus. He found them largely within large endothelial cells and traced them to the lymphocytes in process of being digested. Imamura [16] observed similar bodies in the spleen and lymph nodes, especially the germinal centers, and associated with a general hypolymphocytosis, in animals in which hemolysin had been produced; he traced these bodies to lymphocytes and I agree in this view. Such bodies were first described by Flemming,[17] but since that time they have been studied by a number of observers.

In my work it seems clear that these bodies were derived from large and small lymphocytes and lymphoblasts. They may be said to have developed in three ways: (1) by simple hyperchromatosis of the nuclei, (2) disintegration and metamorphosis of chromasomes after mitosis, and (3) disintegration of a compact homogenous nucleus. In the first case there generally results large irregular, ring or crescent bodies, and in the second a collection of small ball-like masses, both these bodies staining red before disappearing completely. The third change seemed to have affected a nucleus just before division. Many authors have observed these forms of disintegration. Helly,[18] Weiden-

[15] Jour. of Exper. Med., 1898, 3, p. 611.
[16] Nisshin-Igaku, 1916, 6, p. 1871.
[17] Arch. F. Mik. Anat., 1885, 24, p. 50; Arch. f. Anat. u. Entwichlysgeschichte, 1885, p. 221.
[18] Wiener klin. Wchenschr., 1904, p. 639.

reich [19] and others regard the lymphocytes as the most resistant, and it is difficult to distinguish between cells of this kind that are actually dead and others that are still viable. Certainly the lymphocytes seem to multiply more while in the course of degeneration, the toxic action being evident in the course of cell division. We see it in the mulberry nucleus, in which the chromosomes become spherical directly after mitotic division; in the nevt stage they disintegrate into many roundish masses. This occurred in large as well as small lymphocytes, the larger being more numerous in the germinal centers, the small in the pulp. In other cases the nucleus swells up and bursts at the same time as the cell is taking up cocci. The spleen of a guinea-pig that died from subacute streptococcus infection presented a marked degeneration of the cells in the germinal centers with proliferation of macrophages, and in such areas there was an abundance of tingible bodies.

In the liver of a rabbit that died of chronic streptococcus infection were found many cells having peculiar, spoonlike nuclear masses joining in the center, somewhat like a crysanthemum, evidently incomplete degeneration formed.

Peculiar bodies also occurred in the lymph node, intestinal and elsewhere, as well as in the intestinal villi. Such bodies are found also in normal tissues in small numbers, but as a rule the large pyknotic forms in the spleen and lymph nodes are not present.

III. PLASMA CELLS

As described in part I, plasma cells appeared in the spleen, lymph nodes and the submucosa of the stomach, intestines and uterus, soon after the injection of a killed nonhemolytic streptococcus. In the spleen a fairly large number was present as soon as 5 minutes after the injection, and it was easy to trace their development from lymphocytes. After 24 hours, however, the number of plasma cells fell rather promptly. Normally plasma cells occur in the intestinal submucosa, the omentum, lymph nodes, marrow, submaxillary and other glands as well as in ganglions. Thus Jadassohn and others [20] describe them in

[19] Leucocyten u. Verwandte Zellformen, 1911, p. 162.

[20] Jadassohn: Arch. f. Dermat. u. Syph., 1892, 24, p. 216.
Marschalko: Arch. f. Derm. & Syph., 1895, 33, pp. 1 and 241; Centralbl. f. allg. Pathol. u. Anat., 1899, 10, p. 851.
Hedora: Monatschr. f. Prakt. Dermat., 1896, 22, p. 53; Ann. Dermat. Syph., 1895, 6, p. 859.
Unna: Monatschr. f. Prakt. Dermat., 1895, 20, p. 477.
Jovannovics: Ztschr. f. Heilkunde, 1899, 20, p. 159.
Pappenheim: Pappenheim, Virch. Arch., 1901, 166, p. 448.
Maximow: Arch. f. Mik. Anat., 1905 6, 67, p. 680.
Downey: Fol. haemat., 1911, 11, p. 275.
Huebschmann: Verhandl. f. Deutsch. path. Gesell., 1913, 16, p. 110.

[21] Monatschr. f. Prakt. Dermat., 1891, 12, p. 296. Virch. Arch., 1913, 214, p. 320.

the spleen in various mammals including man, but there is no special report on the guinea-pigs in this respect, so for as I can find. In the animals studied so far plasma cells have been found most typically in the rat and mouse: according to Bodora, in man plasma cells may be found either at the periphery of the follicles or in the pulp, these being not wholly typical (pseudoplasma cells). Unna [21] insisted that the plasma cell is a pathologic cell originating from the connective tissue cell, but at the same time he accepted the view of Marschalko that it is a normal cell in the blood-making organs of the mouse. Marschalko urged that the plasma cells in pathologic foci are of hemotogenous origin. Maximow concluded that plasma cells are present sparsely normally and arise in greater numbers in inflammatory conditions. I found on careful examination of the spleen of 5 normal guinea-pigs that a small number of cells were present, resembling very much the typical plasma cell except in respect to the nucleus. In one case plasma cells typical in every respect were found. In the spleen the cells were either grouped or distributed rather irregularly. Usually the cytoplasm did not stain thoroughly and the cells often appeared flat, as described by Maximow. I found a moderate number of such cells in the intestinal wall also. Five minutes after the injection of nonhemolytic streptococci numerous plasma cells would appear, and it is interesting to note that Huebschmann has just found that the spleen is richest in plasma cells in streptococcus infection, especially when it concerns nonhemolytic streptococci.

Staining method.—The tissues were fixed in Zenker's fluid without acid and the sections stained in hematoxylin and eosin as well as with a modification of the gram-Weigart method. For differentiation in the first case hydrochloric acid alcohol was used and in the second case anilin oil and xylol. By thorough differentiation the hematoxylin sections could be made to show the cocci clearly as well as the chromatin network, while a weaker differentiation would give a more characteristic cytoplasm, the nucleus then being stained more diffusely. With the Gram-Weigert stain only the nucleus and the chromatin in dividing cells would be stained. Perhaps the best demonstration of plasma cell was obtained by using both these methods on the same sections. Unna's polychrome methylene blue and the Unna-Pappenheim stain were also useful.

In the spleen plasma cells were found mostly in the pulp, often in small masses. In the malpighian bodies they usually appeared scattered about and in the germinal center were found both large and small types. They were not present where the lymphocytes were packed together tightly. Usually these plasma cells arranged themselves so as to form

some kind of a lumen or space in their midst. The amount of cytoplasm was not always abundant and the shape more irregular than oval or round, due it would seem largely to the spaces which the cells occupied.

Type of Cell.—In the spleen the cell was mostly small (Marschalko's type), although the nucleus often showed only a deeply stained network or small granules rather than a number of peripheral chromatin bodies. Larger types occurred in small numbers, and the cytoplasm was usually rather thin, staining lightly as a rule and the nuclei, composed of chromatin lumps or a network, rarely with definitely arranged chromatin blocks along the peripheral. There was a peculiar cell, rather infrequent, and apparently one of young form; the nucleus consisted of 4-6 deeply stained triangular or quadrangular chromatin blocks, connected by fine strands, but without any membrane. The neighboring edges of these blocks did not always fit well together so that the outline of the nucleus did not form a regular surface. Often there was no nucleolus in these cells. The cytoplasm was often so small that it was difficult to see it, and in most cases it was present at one or both ends of the cell and sometimes abundantly; it was free from granulations. Another type more numerous had a thin nuclear membrane, often defective in places, the chromatin blocks being more numerous and of various forms although mostly trapezoidal with the top projecting inward. As a rule there was a nucleolus in the center, and often abundant cytoplasm, with little granulation. There were numerous intermediate forms between these types, which may be looked on as young cells that develop into the usual type by the production of a thicker nuclear membrane and the rearrangement of the chromatin blocks.

Pappenheim[22] identified the large cell described by Hodora (polyeidocyte) as identical with Schridde's[23] lymphoplastic plasma cells. This cell is of varying shape, has a small amount of cytoplasm and a large vesicular nucleus, usually central, the cytoplasm staining rather lightly. Schridde observed his cell in a study of the hyperplastic tonsil and regarded it as different from the usual plasma cells, which differs from lymphoblasts in having a cytoplasm that stains intensely and in the presence of a perinuclear halo with an eccentric position of the nucleus. He identified his cell with lymphoblasts by demonstrating with his special stain certain perinuclear granules in both these cells. Hertz,[24] discussing the literature, advanced the view that these two types are different, Hodora's cell being of the nature of a splenocyte while Schridde's cell is a lymphoblast. In the spleen of rabbits with an experimental anemia, he found in the interfollicular tissue a large cell which he called splenocytoid plasma cell and which he regards as identical with Hodora's cell. He does not regard these cells as mature, but as capable of developing into the typical plasma cell. In the pulp he found an intermediate type with a more or less oval form, eccentric nucleus, basophil cytoplasm and perinuclear halo, which he regards as a genuine plasma cell in process of formation. He did not find any of Schridde's cells in the germinal center, but he observed a cell with deeply stained cytoplasm, with on the whole a central nucleus, often without a halo, which he regards as an early stage of Schridde's lymphoblastic plasma cell. He calls it plasmacytoid lymphoblast. Evidently Herz believes that various

[22] Fol. haemat., 1911, 11, p. 170.
[23] Zeiglers Beitr., 1907, 41 p. 213
[24] Fol. haemat., 1912, 13, p. 177

larger types of cell may form typical plasma cells, but he does not seem to have observed the latter (Marschalko's type) with transitional forms in the germinal center; he did find, however, large numbers of the small type in the outskirts of the malpighian bodies, together with a smaller number in follicles which seem to have wandered in from the pulp.

Sleginger[25] distinguished four kinds of cells in a leukemic lymph node: (1) those with a small nucleus, with irregularly arranged chromatin, little or no cytoplasm, some having a deeply staining nucleus; (2) those with an oval or round body and eccentric nucleus with a distinct nucleolus; (3) cells with well marked granular cytoplasm, a slight perinuclear halo, the nucleus large, usually eccentric; (4) those with a large body, the vesicular nucleus often assuming a mulberry form and usually with a distinct nucleolus. He observed intermediate forms of all kinds. He does not regard the peripheral arrangement of the chromatin masses as a necessary characteristic of plasma cell and believes that Unna's plasma cell and Marschalko's cell are the same cell under slightly different forms, the small type of plasma cell developing probably from the larger.

Most authors who used hematoxylin, which has a strong affinity for chromatin, have had more or less success in tracing cell development. By means of this stain, and being able to call forth plasma cells at will, I have been able to follow their development in the spleen. Both types appear to be distinct, the large type never changing into the Marschalko type or typical plasma cell. In the germinal center I could distinguish a cell with a round or oval nucleus staining more deeply than in the case of lymphoblasts. The smaller nuclei stained more deeply with coarse chromatin masses, the larger nuclei being more vesicular, a relationship better demonstrated by double staining. Usually these cells had 1-2 and sometimes 3 nucleoli, the larger nuclei being about 8 mikrons wide and 10 or more in length; the cytoplasm was markedly granular, slightly basophil, staining red by the Unna-Pappenheim method, the nucleus nearly neutral and without any halo, as a rule. Mitotic figures were seen frequently. These cells correspond to the plasmacytoid lymphoblasts of Hertz as to character and location and to the Hodora type as to morphology but not location. I saw these cells frequently in the pulp and about the follicles as well as in the germinal centers in pathologic spleen. It may be recalled that Hodora saw them in the normal spleen in man while Hertz found them in the germinal centers in rabbits with experimental anemia and Schridde found them in the germinal centers in the hyperplastic tonsil. I fail to find any such cells in the germinal centers in the normal guinea-pig spleen as Hodora did in man. The lymphoblasts in the germinal center seems to be able from stimulation or otherwise to produce the large type of plasma cell with eccentric nucleus and perinuclear halo. In my experiments I found also large plasma cells in the pulp of the spleen with varying amounts of cytoplasm as well as other variations, all being apparently intermediary or developmental stages of the same cell. Cells corresponding exactly to Schridde's type were relatively few and usually less granular than the ones in the germinal center. Usually these cells were poor in chromatin, the nucleus being vesicular, although some had a fairly rich network of chromatin as well as isolated masses. As in the germinal centers, the smaller the size, the deeper the stain and the larger the size, the more vesicular the nucleus. Sometimes there was a pavement-like arrangement of the chromatin blocks with light lines between them, but a central nucleolus was seen only occasionally, the blocks

[25] Virch. Arch., 1902, 169, p. 428.

being commonly arranged irregularly. Such nuclei are much smaller than the vesicular, the cytoplasm varying in amount, often being abundant. This form of cell appears after mitotic division of large lymphocytes, the young cells first showing deeply stained chromatin masses and being of irregular outline, which later becomes round or oval, when segmentation of the chromatin takes place, often with isolation of the nucleolus. Usually the segmentation occurs irregularly, without the formation of pavement-like nuclei, the chromatin forming a network or irregular masses. In further development the nucleus grows in size at the same time as the chromatin diminishes in amount so that eventually the nucleus becomes vesicular. In cells with abundant cytoplasm there may be a well developed perinuclear halo even in the earlier stages. In this type consequently the tendency to the typical nucleus ('radkern') is not marked at all. In the course of the development of the vesicular nucleus 16-20 small chromatin granules were sometimes seen inside the nuclear membrane.

There are, then, distinct and easily traced stages in the changes of the nucleus after division until it assumes vesicular form.

The Marschalko type of plasma cell appears to develop from small lymphoid cells with a deeply stained nucleus and a pink perinuclear ring and but little cytoplasm. By nuclear segmentation and growth of cytoplasm plasma cells are formed. At first crevices appear in the nucleus along which the chromatin eventually splits to form irregular blocks. At this time there is no nuclear membrane, but this appears a little later, at the same time as a nucleolus forms in the center. The chromatin gradually disappears from the central area and the crevices widen so that a typical nucleus forms to give way later to irregular masses of chromatin, 5-8 in number along the nuclear membrane. In this way more or less atypical plasma cells develop from typical forms. There is no definite rule as to the number of crevices and the chromatin masses vary in size. Atypical cells of this type are common in the spleen while the true Marschalko cell is rather rare. In the early stages there is little cytoplasm, but this develops rapidly, with usually indistinct granulation which becomes more marked later. In the young cells there may be well marked prolongations of the cytoplasm while in the older the margins seem rather thin and even vacuolated.

Authors differ as to whether plasma cells multiply by mitosis or amitosis. I have observed both forms, and the lymphocytic type of plasma cells may arise directly from lymphocytes by division. The large types of plasma cells increase mostly by mitosis; in the germinal centers amitosis of the large cell was not seen, but amitosis of small lymphocytes was common. By amitosis, plasma cells as well as lymphocytes often are divided unevenly, there being much cytoplasm at one pole and hardly any at the other. During and after mitosis both forms of cells may show a distinct granulation in the cytoplasm but this tends to disappear.

It is difficult to understand the origin of the densely stained lymphocytoid cells, whether by immigration or by local production either from lymphocytes by division or as the result of some reactive change in old cells. Judging from the appearances in the liver and the lung these cells would seem to arise from cell division in loco, but I have failed to observe any definite or marked indications of cell division immediately after the injection of the streptococci. It seems that they arise from increased activity on part of the lymphocytes as a result of stimulation by the streptococci. The so-called plasmacytoid lymphoblasts also arise, soon after injection of streptococci, from existing lymphoblasts and form plasma cells by mitosis.

These are my ideas in regard to the development of plasma cells under the conditions outlined. As stated, the lymphocytic small type and the large type, when viewed from the nuclear development, have no connection. Each produces only its kind. As Schlesinger says, we cannot at present tell whether the small lymphocytes are able to produce the large lymphocytes when influenced by special stimuli or otherwise. In the same way the small type of plasma might be conceived as able to produce large types when stimulated to repeated division, but there is no evidence that fully differentiated cells can change from one type to the other. It is possible that the large type may become small and to all intents and purposes be lymphocytes, by loss of cytoplasm and nuclear contraction, but in such a case it would be difficult to account for the changes in the structure of the nucleus, and I have not encountered any apparent transitional forms of this kind. Schridde says that he has not seen lymphocytic plasma cells develop from lymphoblasts, which Pappenheim thinks is possible. I have already stated that there seems to be no connection between the plasmacytoid lymphoblasts of Hertz and the small lymphocytic cells in and about the germinal center, as would be expected from his view. The change in the dense chromatin with course granules to the finer occurs generally in the development of cells. Taking all these points into consideration, I am unable to agree with those that advocate that Marschalko's cell type is derived from the large type of plasma cells. The cells of each type develop as they are, the chromatin undergoing reduction.

It is interesting that all the lymphocytes in the spleen, even in the follicles, should react in the same way after the injection of streptococci. In normal tissue the nuclei of lymphocytes are usually irregular and wrinkled, the chromatin being distributed as irregular granules, but after the injection the nuclear membrane is stretched out in smooth spherical or oval form and the chromatin arranged regularly in coarser blocks as it is in plasma cells, with a purple tinge. In the normal spleen and lymph nodes such cells are seen occasionally. Pappenheim[26] states that while lymphocytes and plasma cells may be isomorphous and isochromatic they nevertheless are heterogeneous elements. Leaving aside the question whether they are heterogeneous or not, it certainly is true that the lymphocytes and plasma cells, after the injection of streptococci, react isochromatically.

In the lymph nodes in various parts of the body, in the walls of the intestines, stomach and uterus, the lymphocytes reacted in the same way as in the spleen at the same time as there was an increase in the number of plasma cells.

Normally, plasma cells are often present in moderate numbers in lymph nodes as well as in the intestinal submucosa, being usually of the small lymphocytic type with a typical nucleus with a halo. After the injection of nonhemolytic streptococci, however, they increased in number and stained more deeply and distinctly, and this was especially true with regard to the uterus and intestine. In the intestinal submucosa plasma cells developed in typical form, with a large amount of cytoplasm, round or oval in shape. In places where the cells were closely packed they were irregular in shape and size. In Peyer's patches and solitary nodes the plasma cells were more of the type of the plasmacytoid lymphoblasts of Hertz or Hodora's pseudoplasma cell. Typical large cells but not with typical nucleus were frequently seen in sections of the intestine free from lymph nodes and lymphocytes were frequently found in the epithelial layer, showing fairly typical plasma cell chromatin but little cytoplasm, the shape being determined apparently by the space they occupied.

[26] Virch. Arch., 1901, 166, p. 424; 1902, 169, p. 372.

Hodora called the plasma cells in the normal spleen polyeidocytes, that is cells with many forms, these cells being round, oval, triangular, pyramidal, spindle shape, etc., the nucleus large, and surrounded by a small amount of weakly staining cytoplasm. Even typical plasma cells with characteristic nucleus and halo, arising after the injection of streptococci, may be of many different shapes. Since Unna characterized the plasma cell as round or oval, this has been accepted as the characteristic shape of the cells, but evidently the form may be greatly modified by the conditions about the cell.

Normally the liver does not contain typical plasma cells, only some small lymphoid cells in the perithelium of the vessels and ducts, especially the latter, with deeply stained nucleus and a small amount of irregular cytoplasm. These cells correspond to Schlesinger's type 1, found in the lymph nodes in leukemia, and they also resemble my younger forms of Marschalko's type, but they do not stain as distinctly and they were few in number. Marschalko did not find plasma cells in the normal liver, while Foa observed them in small numbers in the tissue about the ducts. After the injection of streptococci these cells appeared as plasma cells, being somewaht increased in number after 3 hours and continuing for 96 hours. In the blood vessels of the liver the number of lymphocytes increased and plasma cells often were found among them. Hence the increase of plasma cells in the perithelium may be caused in part by immigration, but most of the cells undoubtedly developed from local production.

The views of the orgin of plasma cells differ greatly. They may be grouped as follows: (1) from fixed connective tissue cell (Unna,[3] L. Ehrlich[17] and others; (2) from lymphoid cells of either blood or tissue origin or both, including mononuclear leukocytes and perithelial cells, this view being supported by many investigators (Marschalko, Krompecher,[28] Maximow, Enderlen-Justi,[29] etc.); (3) from lymphocytes and lymphoid cells as well as fixed connective tissue cells (Pappenheim, Almgvist,[30] and others). We see that the opinions appear to favor the origin of plasma cells from lymphocytes or lymphoid cells. The question now seems to be whether the lymphoid cell is a highly differentiated fixed connective tissue cell or a real lymphocyte. Leaving aside for the moment the question whether these cells are of blood or tissue origin, the results of my work indicate that the plasma cell develops from small lymphoid cells as well as from large lymphoblasts, in the spleen and other lymphoid tissues, as the result of a special stimulation.

Pappenheim asserts that the Morschalko type is not derived from immigrated lymphocytes but rather from local tissue cells, possibly from myeloid forms, while Unna's type is of fibroblastic nature. I wish to point out with special emphasis that in my experiments the lympho-

[27] Virch. Arch., 1904, 175, p. 198.
[28] Zeigler, Beitr., 1898, 24, p. 163.
[29] Deut. Ztschr., f. Chir., 1901-2, 62, p. 82.
[30] Arch. f. Dermat. u. Syph., 1901, 58, p. 91.

cytes in the spleen and elsewhere showed the same reaction of the chromatin on the injection of streptococci, but that the cells in the marrow failed to react, indicating a difference in the nature of the cells, and this was found to be the case even though the cells in the marrow multiply rapidly. In the course of their multiplication the cells in the marrow had no tendency to form characteristic plasma cell nucleus. Schridde made a similar observation with a special stain. These observations do not harmonize with the suggestion of Pappenheim that possibly plasma cells are derived from myeloid cells. In my experiments plasma cells appeared within 5-10 minutes in the spleen, liver and other organs, so quickly that it does not seem likely that they were immigrated cells from the blood, although the appearance of plasma cells in the blood later was a fairly regular occurrence. At the same time these cells may have developed both from preexisting lymphoid cells and from newly immigrated lymphocytes from the blood. As stated, Hertz regards his splenocytoid plasma cells as coming from splenocytes; in my experience the large type was often seen in the capillaries and sinuses of the spleen and resembled somewhat the splenocytes. However, plasma cells are not associated in phagocytosis of streptococci and red blood corpuscles, whereas splenocytes are very active in that respect. In experiments in which I injected India ink before or after streptococci, no phagocytic plasma cells were seen, even when granules were lying around plasma cells. Most authors agree that the plasma cell is not phagocytic but Marschand [31] describes the phagocytosis of lepra bacilli by these cells. Goldmann [32] distinguishes plasma cells from other cells because they are not stained vitally pyrrhol blue and Kiyono [33] separates them from other cells because they do not stain vitally with carmin. Consequently, it is difficult to accept the view of Hertz that plasma cells are derived from splanocytes. It is more likely that plasma cells develop from lymphocytes rather than from fixed connective tissue cells.

IV. LEUKOCYTOSIS FOLLOWING THE INJECTION OF KILLED NONHEMOLYTIC STREPTOCOCCI

In the last years of the previous century the changes in the number of leukocytes in the blood following the injection of protein substances, bacteria and their products, as well as organ extracts, were studied

[31] Verhandl. d. Deutsch. Path. Gesell., 1913, 16, p. 5.
[32] Beitr. f. klin. Chir., 1909, 64, p. 192.
[33] Folia haemat., 1913, 15, p. 282; Die vitale Karminspeicherung, Jena, 1914; Nisshin-Igaku, 1914, 4, p. 917 and 1113.

extensively. Lowit[34] expressed the idea that the leukocytes in the blood are easily destroyed as the result of the injection of foreign substances and that this destruction is followed by an overflow of leukocytes from the blood-producing organs, the chemotatic influence of the injected substances being of little or no importance. According to this view, a primary leukolysis occurs followed by a secondary leukocytosis. Lowit regarded the leukocytosis following blood letting, in inflammation, in digestion, etc. as the expression of an overcompensation following leukocytic destruction. Schulz,[35] however, observed that after the injection of various substances the leukocytes would be distributed irregularly in the body, and on this account he could not accept the view of hypoleukocytosis and hyperleukocytosis. Goldscheider and Jacobs[36] observed that in the stage of leukopenia the leukocytes gather in the internal organs, especially the lungs, and they attributed this to chemotactic action of the substances infected and explained the subsequent hyperleukocytosis on the same basis. In 1904 Arneth[37] noted that the neutrophil leukocytes undergo morphologic changes in infectious diseases, and he classified these leukocytes into 5 types according to the number of the nuclear lobules. He advanced the view that the neutrophil leukocytes were destroyed as a result of the infection. In my experiments with India ink and with physiologic salt solution I noted usually a slight degree of stimulation in the lung during the period of leukopenia. This usually lasted from 10 to 30 minutes after the injection, sometimes as long as 60 minutes and was then followed by a leukocytosis that reached its maximum after 3 to 4 hours. When large quantities of ink were injected there resulted a more marked destruction of cells without leukocytosis, the pseudo-eosinophil cells being replaced by a new immature cell some time after the injection, showing distinctly that destruction, as well as chemotactic influence, play a significant part. After the injection of killed nonhemolytic streptococci the distribution of leukocytes in the early stages corresponded to that described by Goldscheider and Jacob, large numbers gathering in the lung; this fact accounts in some degree for the leukopenia, and the changes in the bone marrow indicate a rapid mobilization and proliferation of cells. Muir[38] observed a marked proliferative progress in the marrow in

[34] Studies zur physiologie und pathologie des Blutes und der Lymphe, Jena. 1892.
[35] Deutsch. Arch. f. klin. Med., 1893, 51, p. 234.
[36] Ztschr. f. klin. Med., 1894, 25, p. 373.
[37] Die Neutrophilen Weissen Blutkörperchen, Jena, 1904.
[38] Jour. of Path. and Bacteriol., 1900-01, 8, p. 161.

hyperleukocycosis with decrease of polymorphonuclear cells while Rubinstein[39] found an increase of lymphoid cells, myelocytes and transitional forms, the polymorphonuclears showing a decrease. These authors interpret the decrease of polymorphonuclears in the marrow in this instance as the result of rapid transfer of cells to the blood. Up to this time there was usually not observed any accumulation of polymorphonuclears in the marrow in infection, spontaneous or exeprimental, but this probably is due to its manifest nature and its occurrence shortly after the introduction of the foreign substances.

I shall discuss the changes in the leukocytes in the blood after the injection of killed nonhemolytic streptococci. Some of the questions to be solved if possible are the fate of the leukocytes that are destroyed and whether new and special forms of cells would appear as after the injection of India ink. The question as to how long the cocci remain in the blood stream, inside as well as outside the leukocytes, also presents itself for consideration.

In the experiment another strain of nonhemolytic streptococcus was injected. Otherwise the same procedure was followed and blood was taken from the right ventricle of the heart at various intervals after the injection with a fine needle and syringe. Fresh counts were made and also stained preparations. The total number of leukocytes was obtained by getting the average of ten large square fields from both sides of Turks' double chamber chemocytometer. Wright stain was used for differentiation and hematoxylin and eosin stains were used to determine Arneth's counts. The same suspension of streptococci was used throughout the experiment, the suspension being kept in the icebox and warmed to about 38 C. each time before injection.

The results obtained in these experiments were not consistent. Apparently unknown factors requiring further study played a part. The observations recorded in Table 3 were, however, so consistent as to require no further observations.

As to the total number, it gradually increased reaching a maximum about 3 hours after injection and then fell gradually in the case of guinea-pig 1 in which there was no fall immediately after the injection as expected. In guinea-pig 2 the number increased rapidly and returned to normal after 6 hours. In guinea-pigs 3 and 4 death occurred at the end of 1 hour when there was a distinct leukopenia. These animals remained on the holder for 30 minutes after the injec-

[39] Ztschr. f. klin. Med., 1900-01, 42, p. 161.

tion. Lowit noted a decrease in the number of leukocytes as soon as the animal was placed on the holder but did not examine the blood of the heart. He also found that lowering of the temperature of the body reduced the number of leukocytes and Goldscheider and Jacob believed that such lowering accounted for the fall in the leukocytes rather than placing the animal on a holder. The other animals in my experiments showed no decrease and very little increase in the period concerned. When we consider the preservation of the suspension of the streptococci, the possibility arises that products may have passed into solution that were toxic for the animals, and on this account the cocci were washed before injecting guinea-pigs 5 and 6, the results not differing very much from those obtained in animals 1 and 2. In none of these experiments except in that performed on animal 4 was there any marked fall in leukocytes in the heart blood within 10-30 minutes after the injection, but there was a more or less irregularly occurring increase in the number.

The differential count established a decrease in the large mononuclear leukocytes throughout the period under observation and of the polymorphonuclears for a short time after the injection when the number rose again reaching normal in about 24 hours. The lymphocytes, however, increased in number soon after the injection in most cases, and the polymorphonuclears increased after 3 hours in every case, returning to normal at the end of 24 hours. The fact that there was no diminution of leukocytes immediately after injection may have been due principally to increase in the number of lymphocytes. The polymorphonuclears and large mononuclears were usually fewer than normally 10 minutes after the injection. In 3 hours, however, the polymorphonuclears had increased beyond their normal number while the large mononuclears tended rather to decrease in number. In my work with India ink I observed that the large mononuclears do not usually enter the blood stream as such but develop mainly from smaller cells as asserted by Gulland and others, and that one or two days are required for their full development when their phagocytic activity is at its height. They may accumulate in the lungs shortly after the injection as the polymorphonuclears do. If not destroyed in the lungs and if they reenter the circulation, the number of mononuclears should be restored promptly, but their number continued low for 24 hours. Where do the polymorphonuclears come from when their number is increased beyond the normal? Are they newly formed

cells produced in the marrow, or do they come from the lung? The leukocytes containing bacteria leave the lungs early and this consequently may be the cause of the rise of such cells in the blood. The histologic examination showed that at this time polymorphonuclears accumulate in the internal organs, such as the liver and spleen, and the increase of these cells in the blood in the right ventricle suggests that they come not only from the lung but also from the blood-forming organs. On the whole, it seems to me that leukopenia and hyperleukocytosis may be caused by the same factor, such as a local positive chemotaxis leading to accumulation in the lung and later in the liver and spleen, as shown in this work, creating a demand for leukocytes from the blood-forming organs and a diminution of leukocytes in the blood. The chemotactic influence affects not only the reserve leukocytes in the marrow, but also stimulates the myelocytes to produce new cells and thus hyperleukocytosis develops. As the streptococci that have been taken up by leukocytes undergo disintegration, substances are set free that may have chemotactic effects and stimulate cell production. If the leukocytes are withdrawn from the blood by the presence of widely spread substances, all organs should receive leukocytes, but my results show that leukopenia as well as hyperleukocytosis are associated with an irregular distribution of the leukocytes owing to their being attracted to places where the streptococci localize. Thus, as already stated, there is a large number of polymorphonuclear leukocytes in the spleen normally, but immediately after the injection of streptococci there was a distinct diminution of leukocytes in the spleen. This occurred also in the bone marrow. The leukocytes undoubtedly pass to the lungs where streptococci accumulated quickly after the injection, as shown especially by the increased number of leukocytes in the earliest stages after the injection. Buchner[40] and others have shown that bacterial protein and cellular protein have a positive chemotactic effect, and it would seem reasonable to believe that the killed streptococci when injected into the blood may liberate similar substances. If leukocytes and other cells undergo destruction, chemotactic substances may be set free and give rise to leukocytosis. In the case of the animals injected with streptococci, cocci and leukocytes accumulate in the lung very quickly and in this respect the result differs greatly from the result of the injection of a simple foreign body, such as a cinnabar without gelatin. It may be pointed out that

[40] Berl. klin. Wchnschr., 1890, 27, p. 673; ibid., p. 1084.

TABLE 3

Analysis of Leukocytes

Guinea-Pigs	Leukocytes	Before Injection	Time After Injection: 10 Minutes	30 Minutes	1 Hour	3 Hours	6 Hours	12 Hours	24 Hours	48 Hours	Remarks
1. 350 gm. injected with 2.3 c c. suspension 2 days old	Total number	11,976	13,280	14,780	17,460	23,840	17,422	18,700	10,320	8,460	
2. 300 gm. injected with 2 c c. suspension 3 days old	Total number	5,780	10,110	11,500	10,660	7,140	5,480	5,500	6,000	......	A moderate number of free cocci at 10 minutes, none at 30
	Mononuclears, %	20.6	10.9	8.2	15.2	10	8.7	16.6	6.2		
	Lymphocytes, %	61	75	75.3	58.7	30.4	31.9	29.1	69.1		
	Polymorphonuclears, %	15.2	13.2	15.5	23.4	58.4	59	53.6	24.3		
	Other cells, %	3.1	0.8	0.9	2.6	1.1	0.4	0.6	0.3		
	Arneth's count I	19	......	3	13	44	40	19	10	......	At 3 hours 98% pseudos replaced by cells which died in about 20 hours
	Arneth's count II	10	......	12	18	44	38	34	33		
	Arneth's count III	10	......	28	37	11	20	36	37		
	Arneth's count IV	28	......	34	25	1	2	10	16		
	Arneth's count V	35	......	23	7	0	0	1	4		
	Phagocytic mononuclears, %	.....	8.7	5.4	2.8	0	0	0	0		
	Phagocytic polymorphonuclears, %	.....	44.2	22.2	11	2.6	0	0	0		
3. 280 gm. injected with 2 c c suspension 4 days old	Total number	6,466	7,590	5,820	2,120	......	......	......	......	......	Moderate number of cocci among blood platelets at 10 minutes, hardly any at 30
	Mononuclears, %	17	17.6	11.1	12.7						
	Lymphocytes, %	37.8	53.4	44.4	38.7						
	Polymorphonuclears, %	42	26.1	40	43.9						
	Other cells, %	3.1	2.8	4.4	4.6						
	Phagocytic mononuclears, %	.....	9.3	4.4	9						
	Phagocytic polymorphonuclears, %	.....	23.3	7.7	28						
4. 250 gm. injected with 1.5 c c suspension 5 days old	Total number	4.884	2,820	2,140	1,253	......	......	......	......	......	A fair number of cocci among blood platelets at 10 minutes, only a small number at 30 minutes
	Mononuclears, %	30.2	18.7	9							
	Lymphocytes, %	37.8	49.9	61.8							
	Polymorphonuclears, %	29.3	27.3	22.7							
	Other cells, %	2.6	4	6.4							
	Phagocytic mononuclears, %	.....	2.4	0							
	Phagocytic polymorphonuclears, %	.....	23.3	40							

5. 260 gm. injected with 1.5 c c suspension 6 days old after washing the cocci	Total number	4,300	6,500	5,520	5,520	5,350	2,420	3,060	3,680	......	Moderate number of free cocci among platelets at 10 m., none at 30. No peculiar cells after 1 hour; at 3 hours all pseudoeosinophils were replaced by peculiar cells; at 12 hours cells mostly of normal type with some transitional forms; at 24 hours peculiar cells rarely seen
	Mononuclears, %	34	13.9	12.4	12.1	17.7	15.4	10.3	14.4		
	Lymphocytes, %	21.6	54.7	46.7	58.3	23.6	30.9	41.3	45.3		
	Polymorphonuclears, %	36	27.8	34.7	21.2	52.9	47.7	47.1	34.1		
	Other cells, %	8.3	3.5	6.1	8.3	5.7	5.9	1.2	6.1		
	Phagocytic mononuclears, %	.....	0	0	0	0	0	0	0		
	Phagocytic polymorphonuclears, %	.....	23.2	29.6	21.4	2.7	0	0	0		
	Phagocytic eosinophils	.....	2.1	0	0	0	0	0	0		
	Arneth's count I	3	......	......	......	43	41	16	19		
	Arneth's count II	11	...	.....	...	44	36	32	31		
	Arneth's count III	21	......	......	......	11	20	38	40		
	Arneth's count IV	27	......	......	......	2	3	12	9		
	Arneth's count V	38	......	......	......	0	0	2	1		
6. Suspension 7 days old	Total number	5,420	7,400	6,380	6,850	7,800	3,984	......	4,200	......	Only a small number of free cocci at 30 min. No special form of polymorphonuclear at 1 hr., but at 3 hrs. 95% were special type and at 6 hrs. transitionals and small type still abundant, but at 24 hours nearly all cells were of the usual type
	Mononuclears, %	15.3	......	4.2	4.4	2.1	1.5	......	6.3		
	Lymphocytes, %	55.8	......	74.4	69.6	55.7	30.8	......	65.3		
	Polymorphonuclears, %	15.3	......	20.7	24.3	40.5	66.6	......	27.4		
	Other cells, %	0	......	0.6	1.6	1.6	0	......	0.9		
	Phagocytic mononuclears, %	.....	......	0	0	0	0	......	0		
	Phagocytic polymorphonuclears, %	.....	......	34.5	24.4	3.5	1.2	......	0		
	Phagocytic eosinophils	.....	......	3.1	0	0	0	......	0		
	Arneth's count I	2	......	4	11	37	26	......	7		
	Arneth's count II	9	......	15	17	41	40	......	21		
	Arneth's count III	32	......	36	45	20	25	......	31		
	Arneth's count IV	17	......	30	22	2	7	......	30		
	Arneth's count V	18	......	15	5	0	2	......	11		

the loss of streptococci in suspension of the ability to stain by Gram's method may be associated with the liberation of chemotactic elements. Certainly during the course of phagocytosis, chemotactic substances may be freed by cell destruction and otherwise. Taking these factors into consideration, it seems safe to conclude that the changes in the distribution and number of leukocytes are the result of chemotactic effects.

Among the interesting changes that follow the injection of killed nonhemolytic streptococci is the production of a new kind of cell. Not only were the pseudoeosinophils displaced to the left in Arneth's table, but they multiplied rapidly. With the immature forms, including Papenheim's metamyelocytes, similar to those that I noted after the injection of India ink, this change occurred as quickly as three hours after the injection. In animals 2 and 5 this blood picture continued until about the sixth hour; by the twelfth hour the pseudoeosinophils had decreased and a smaller type with transitional forms then predominated, and after 24 hours normal conditions were practically reestablished. In animal 6, however, the return to the normal seemed to have taken place more rapidly. This cell also took up cocci. The changes in the blood correspond well to the changes in the marrow. As stated, the myelocytes during the early stages after the injection were enlarged and there appeared well lobulated polymorphonuclears in the marrow after 24 hours and later. Pappenheim [41] classifies the myelocytes in young mother cells and young micromyelocytes, the latter developing from the former and becoming transformed into the usual polymorphonuclear cells; the mother myelocytes appear in the blood not only in chronic myelogenous leukemia, but in acute cases polymorphonuclear giant leukocytes are found in the blood. The large myelocytes in the marrow in guinea-pigs injected with streptococci corresponded to the mother myelocytes and polymorphonuclear giant leukocytes were also seen. Pappenheim and Szecsi,[42] as well as Isaac and Moeckel,[43] observed similar cells after saponin injection. We see then that within 3 hours after the injection the reserve cells in the marrow became exhausted and that there appeared a large myelocyte and immature cells in the blood in large numbers. A complete change in the morphology of the leukocytes took place in 3 hours, showing what a large number of cells were

[41] Atlas d. Menschlichen Blutzellen, 1911-12, Suppl. p. 96.
[42] Fol. Haemat., 1912, 13, p. 25.
[43] Ztschr. f. klin. Med., 1911, 72, p. 232.

demanded, how rapidly they perished and how freely their loss was compensated, the ability to supply normal cells being exhausted and immature cells supplied instead. It is probable that the phenomena observed in connection with the leukocytes also took place with respect to the endothelial cells of the liver and spleen, as indicated by the irregular changes observed in their shape, staining and distribution.

In normal guinea-pigs the nuclei of the leukocytes are much more lobulated than in man and they belong principally to classes 3, 4 and 5 in Arneth's scheme. Thirty minutes after the injection and more noticeably one hour later there was a displacement to the left; at 3 and 6 hours after the injection this position was entirely reversed, class 5 being lacking in most cases. As the table shows, classes 1 and 2 constituted 78 to 87% at 3 hours and 66 to 77% at 6 hours. In all cases the maximum was reached at 3 hours and in no case was the normal position regained after 24 hours; animal 6 showed the most rapid return to normal. Arneth was hardly able to recognize any changes from the normal in digestive leukocytosis; after baths and even in infectious diseasees there was only a slight displacement if any in light cases My tables shows that even when many bacteria are introduced at once so that many cells are required for defense, the bone marrow is still able to furnish leukocytes of the usual type, provided it is in normal condition. The marrow always has a considerable reserve of polymorphonuclear leukocytes. When the demand is urgent and continuous the reserve is soon exhausted and younger forms are sent out with a corresponding displacement to the left in Arneth's scale, and eventually special types of cells, such as the large polymorphonuclears with basophil granules, appear. Apparently it takes a little time after cell division before a polymorphonuclear leukocyte reaches its normal and usual form. It is possible that immature cells, which usually are found in the marrow under such conditions, may ripen in the blood, although it appears that usually such types are soon destroyed. Frequently I encountered large cells with fantastically shaped nuclei, sometimes partly segmented, resembling megakaryocytes, and these no doubt are extreme examples of abnormal newly formed leukocytes. The changes found in the leukocytes after the injection of streptococci may be due to fluctuations and changes in chemotactic substances in the blood. We do not know the fate of such substances, whether they are discharged by certain organs or assimilated; we know only that

the cocci disappear completely within 72-96 hours after the injection with gradual changes in form and staining reaction, mostly within cells and that at the same time many cells undergo disintegration. In the meantime, the leukocytes in the blood rise and fall as the balance between destruction and production varies and in any case the appearance of immature leukocytes indicates an acute demand either on account of a general destruction of leukocytes or on accout of their being attracted in large numbers to some particular place. The rapid changes in the leukocytes soon after the injection shows how powerful the chemotactic influences at that time were.

In view of what has been stated, it appears that in infectious diseases a hyperleukocytosis or a chronic leukopenia, with normal polymorphonuclears, indicates that there is a reserve supply of leukocytes in the marrow; in other words, that the prognosis seems good; but an acute hyperleukocytosis with many immature leukocytes with marked displacement to the left indicates that the demands on the marrow are approaching the limit of its powers. This would be still more strongly indicated in case an acute leukopenia, affecting especially the polymorphonuclears, should develop. At this point it may be suggested that in acute infectious diseases careful examination of the blood by Arneth's method is indicated, especially when vaccine treatment is used, because the injection of killed bacteria or their products at short intervals may serve to increase the exhaustion of leukocytes.

In these experiments it was assumed that the streptococci that were injected would remain free in the blood for about 30 minutes, but it was found that they quickly became enclosed within masses of platelets and apparently rapidly reduced in number. As the table shows, streptococci were found within leukocytes in most cases for as long as 3 hours; in animal 6 there were cocci within the leukocytes 6 hours after injection. It is possible that cocci remain free in the blood in small numbers for a longer time because so many phagocytic cells were attracted to certain organs; even derivatives of mother myelocytes contained cocci. In some of the animals mononuclear cells were found to contain cocci. Eosinophil leukocytes, which do not take up India ink granules, frequently contained cocci.

SUMMARY

On injection of homogenous suspensions of nonhemolytic streptococci into the jugular vein of guinea-pigs, the cocci first accumulate principally in the lung, a comparatively small number only passing

through this organ into the general blood stream, whence they are taken up by endothelial cells in the liver and by endothelial and other cells in the slpeen. Injection into the left ventricle and into the portal vein also resulted in cocci collecting to some extent in the lung. This accumulation of cocci in the lung seems to be largely mechanical and the result of the formation of masses of cocci in the blood on the one hand and the narrowness of the capillaries in the lungs on the other hand.

In the lung the cocci are taken up principally by polymorphonuclear leukocytes and to a much less degree by mononuclear and endothelial cells. The rapid collection of leukocytes in the narrow pulmonary capillaries hinders the dispersion of bacteria into the blood current. The leukocytes are actively phagocytic and within about 10 minutes after the injection apparently all the cocci are taken up; in from 30 minutes to 1 hour the phagocytic leukocytes leave the lungs, the majority going to the liver and spleen, where there is a great increase in such cells up to about 6 hours after the injection. Many cocci are digested in the cells in a short time, but it may require from 72 to 96 hours before all the cocci disappear. During this time the cocci in the cells become smaller, staining irregularly, and finally forming red granules.

The phagocytic action of endothelial and mononuclear cells in the lung is not marked and does not constitute a factor of any great importance in the elimination of the cocci, but in the liver the endothelial cells are very active in phagocytosis, more so by far than the cells of the spleen. On account of its volume and the phagocytic activity of its endothelial cells the liver must be regarded as the most important place of elimination of the bacteria, the spleen probably being next in importance. Of the leukocytes in the blood the polymorphonuclears are by far the most active and undoubtedly play an important part in the digestion of the cocci. Comparatively speaking, the mononuclear and eosinophil leukocytes are less active.

The fixed cells of the marrow are not as active in phagocytosis as those of the spleen, but in the marrow an active supply of new leukocytes develops very soon after the injection, that is within 5 to 10 minutes, and in 1 to 3 hours there is abundant evidence of new formation of leukocytes from myelocytes.

The endothelial cells of the blood vessels elsewhere than in the organs and tissues mentioned do not appear to act as phagocytes

except in the case of the suprarenals and the cortex of the kidney, especially the glomeruli, but the activity here does not seem to be marked. The salivary glands, pancreas and lymph nodes do not seem to participate actively in the elimination of the cocci, which are found only within the leukocytes in these tissues. There is no special relation between the cocci and the walls of the stomach. The endothelial cells of the capillaries of the liver, the lung and also to some extent of the suprarenals and kidneys seem to take on increased activity soon after the injection. This was marked particularly in the liver in which the endothelial cells changed their shape and increased more or less in size, the nuclei staining deeply, the changed cells having a rather irregular distribution. In the spleen there was general proliferation of the reticular cells and of the cells in the malpighian bodies; here a degenerative form of lymphocytes with pykosis or karyorhexis appeared. In the marrow the megakaryocytes also fell into pyknosis on the one hand, others showing mitosis. Soon after the injection plasma cells of various types appeared in the spleen, lymph nodes, and the submucosa of the gastrointestinal tract, uterus, and in other places (see part 2).

The outstanding features of the cellular reaction after the intravenous injection of nonhemolytic streptococci are the accumulation of phagocytic polymorphonuclears in the capillaries of the lung, the liver and spleen; the great leukocytogenic activity of the marrow; and the phagocytic activity of the endothelial cells of the liver and spleen, and of the polymorphonuclears in the blood.

In the spleen were found degeneration forms of the nucleus (Flemming's tingible body), derived in this case from small and large lymphocytes by pyknosis and karorrhexis. Pyknosis apparently resulted from a perinuclear or net hyperchromatosis while karyorrhexis affected chromosomes after cell division, the first step being the formation of the so-called mulberry nucleus. In some cases, however, cells disintegrated that were not in process of division, the nuclei undergoing swelling and assuming budded forms at the same time as the cells contained cocci. These appearances coincide with the appearances that develop after injection of India ink. In the pyknotic group the cells were usually situated within large phagocytes. Irregular disintegration forms appeared also in lymph nodes, intestinal walls, the liver and elsewhere.

In the spleen of the normal guinea-pig there often is a small number of plasma cells; these cells sometimes have a typical plasma cell nucleus, but more usually the cell body is rather flat and does not stain well. After the injection of nonhemolytic streptococci, however, plasma cells became abundant and appeared typical in shape and staining reaction, most of them being of the lymphocytic type (Marschalko), but a larger type also appeared in the pulp and in the germinal centers. The plasma cell of the former type was present in small numbers in the follicles and germinal centers. The large type corresponds to the plasmacytoid lymphoblast of Hertz and to Hodora's pseudoplasma cell, but not to Schridde's lymphoblastic plasma cells. In the pulp were various forms of this large type but there were generally few well developed Schridde's cells.

Cells of the Marschalko type developed from lymphoid cells, the nuclei of which stain homogeneously and separate into segments, without nuclear membrane. In the course of this segmentation the nucleolus often comes to occupy the center. Later a nuclear membrane appears and the segments arrange themselves along its inner surface, a more or less typical nucleus being produced. This change appears to represent a reaction on the part of the lymphocytes.

Plasma cells of the small type multiply by mitotic as well as by amitotic division, while a large type multiplies principally by mitosis.

The chromatin in the large type is scanty, but a short time after division it assumes its usual arrangment; in both types of plasma cells the younger have more chromatin in the form of blocks and masses that stain deeply. As the cell grows the nucleus becomes more vesicular in appearance. There is no indication that these two types of plasma cells change from one into the other.

The plasma cells in the spleen have a relatively small amount of cytoplasm and may assume various shapes while those in the intestine and uterus have rather more cytoplasm and are round or oval, due to the mechanical conditions present.

As indicated, the plasma cells that appear in the spleen and other organs after injection of nonhemolytic streptococci are not the result of immigration but of local production. In the perithelium of the vessels of the liver and especially about the bile ducts occur normally lymphoid cells and 3 hours after the injection of streptococci these cells are increased, this process continuing for about 96 hours.

Undoubtedly the plasma cells sometimes observed in the blood either of the heart or of other organs were in the course of migration. It would seem reasonable to conclude that in local inflammation and tumors plasma cells may develop from existing perithelial lymphoid cells, as well as from immigrated lymphocytes.

The plasma cells did not take up any streptococci, ink granules or blood pigment, while splenocytes had strong avidity for such particles. This is in agreement with the observations of others and particularly with results of Goldmann's experiments with pyrrol blue and of Kiyono's vital stain with lithium carmin. The opinion of Hertz that plasma cells (Hodora type) are derived from splenocytes is not supported by my observations, and the indications are further that plasma cells do not come from the fixed cells of connective tissue.

Generally speaking, the lymphocytes in the spleen, lymph nodes and other organs present the same reactions on the part of the chromatin as the plasma cells, changing from being shrunken to being stretched, from staining indistinctly to staining distinctly, and with my double stain the coarser chromatin particles assumed a purple tint, particularly those along the nuclear membrane, especially in plasma cells.

Injections of a suspension of killed nonhemolytic streptococci, kept for 5 days, and of suspension of washed cocci, caused a varying degree of hyperleukocytosis in the blood of the right ventricle as early as 10 minutes after injection. At the same time, there accumulated in the lungs large numbers of leukocytes so that one would expect a general leukopenia. The hyperleukocytosis continued for about 3 hours, and then disappeared more or less gradually. The latter period of this hyperleukocytosis corresponds to the period when the number of leukocytes in the lungs decreases while in the liver and spleen the number increases. These facts indicate that not only are leukocytes attracted from the blood to the places where the cocci accumulate in largest number, but also and very promptly from the blood forming organs. The negative chemotaxis that Goldscheider and Jacob advanced as an explanation of the accumlation of leukocytes in the lungs and of the leukopenia cannot be accepted; a more reasonable explanation is that a local positive chemotaxis is produced immediately after the injection by the bacteria, as well as by the products of cells that undergo disintegration.

The large mononuclear leukocytes decreased in number immediately after the injection and were not restored within 24 hours, an observation that agrees with the view that these leukocytes do not come into the blood as they are, but rather develop in the blood, and that after having reached full growth they are destroyed easily and quickly. The lymphocytes increased promptly and then gradually decreased to normal again in about 24 hours or so. The polymorphonuclears decreased promptly but in 3-12 hours there was a marked increase and at the end of 24 hours the normal number was reached.

Disarrangement of the normal Arneth's scale began promptly after the injection and reached its maximum in about 3 hours when classes one and two constituted 78-88%. At 6 hours classes 1 and 2 constituted 66-78%. There then ensued a gradual return, but normal conditions were not regained fully at the end of 24 hours. The normal type of cell was replaced within 3 hours by an abnormally large polymorphonuclear leukocyte (mother myelocyte and metamyelocyte, Pappenheim), and in 3 experiments these cells at the end of 3 hours constituted 98, 100, and 95%. This type vanished almost wholly in 24 hours.

This condition in the blood harmonizes well with the result of microscopic examination of the marrow and appears to be the result of an exhaustion of the reserve cells in the marrow and repeated rapid cell division.

The accumulation of polymorphonuclear leukocytes in the lung may be a factor in the general leukopenia that developed after the injection of various bacteria and other substances, but it is not the only factor. Leukocytes may be destroyed so rapidly that in spite of rapid mobilization from the blood-forming organs the normal type of polymorphonuclears may be exhausted within 3 hours or so and replaced by a special type of young cell.

The lymphocytes in the blood from the right ventricle were found to increase from the first after the injection, and in this respect my results agree with those of Lowit.

Only a few phagocytic leukocytes were found in the blood 3 hours after the injection, and in 6 hours such leukocytes seemed to have disappeared entirely and no free cocci could be seen. Eosinophil cells also took up cocci.

In infectious diseases hyperleukocytosis or leukopenia is not a dangerous sign, provided the blood presents the normal leukocytic picture,

but a marked displacement to the left in Aarneth's count, especially when associated with diminution in the number of leukocytes, is a sign of danger, as it means exhaustion of the leukocytogenic function of the marrow.

In severe acute infections the blood should be examined carefully according to Arneth's method, before as well as after the injection of vaccine in case vaccine treatment is used. Repeated injections of vaccines at short intervals may produce a more or less severe exhaustion of the marrow.

Explanation of Plate I

Fig. 1.—Various stages of plasmacytoid lymphoblasts (Hertz) in germinal center with a few small cells of the Marschalko type; spleen, 30 minutes after injection. Unna-Pappenheim stain. Figures 1 and 2, Leitz ocular 4, Spencer objective. oil immersion 1.8; other figures same objective but ocular Leitz 6. All figures outlined by aid of Zeiss apparatus.

Fig. 2.—Development stages of nucleus of large plasma cells after mitosis. A1 and A2, at the right, young Schridde type; A2, left, still younger; B, usual Schridde type; spleen, 5 minutes after injection; hematoxylineosin and Gram-Weigert stain.

Fig. 3.—A, Schridde type in pulp; B. abnormally developed vesicular Hertz type with reguarly arranged chromatin granules inside of nuclear membrane; hematoxylin-Gram double stain.

Fig. 4.—Developmental stages, Marschalko cell. A, lymphoid cell; B, faint crevices; C, developed crevices, no nuclear membrane; D, appearance of nuclear membrane; E, usual Marschalko type in spleen.

Fig. 5.—A, various Karyorrhectic degeneration forms of small lymphocytes 6 to 12 hours after injection; B. special peculiar cells in liver of rabbit with chronic septicemia; hematoxylin-eosin stain.

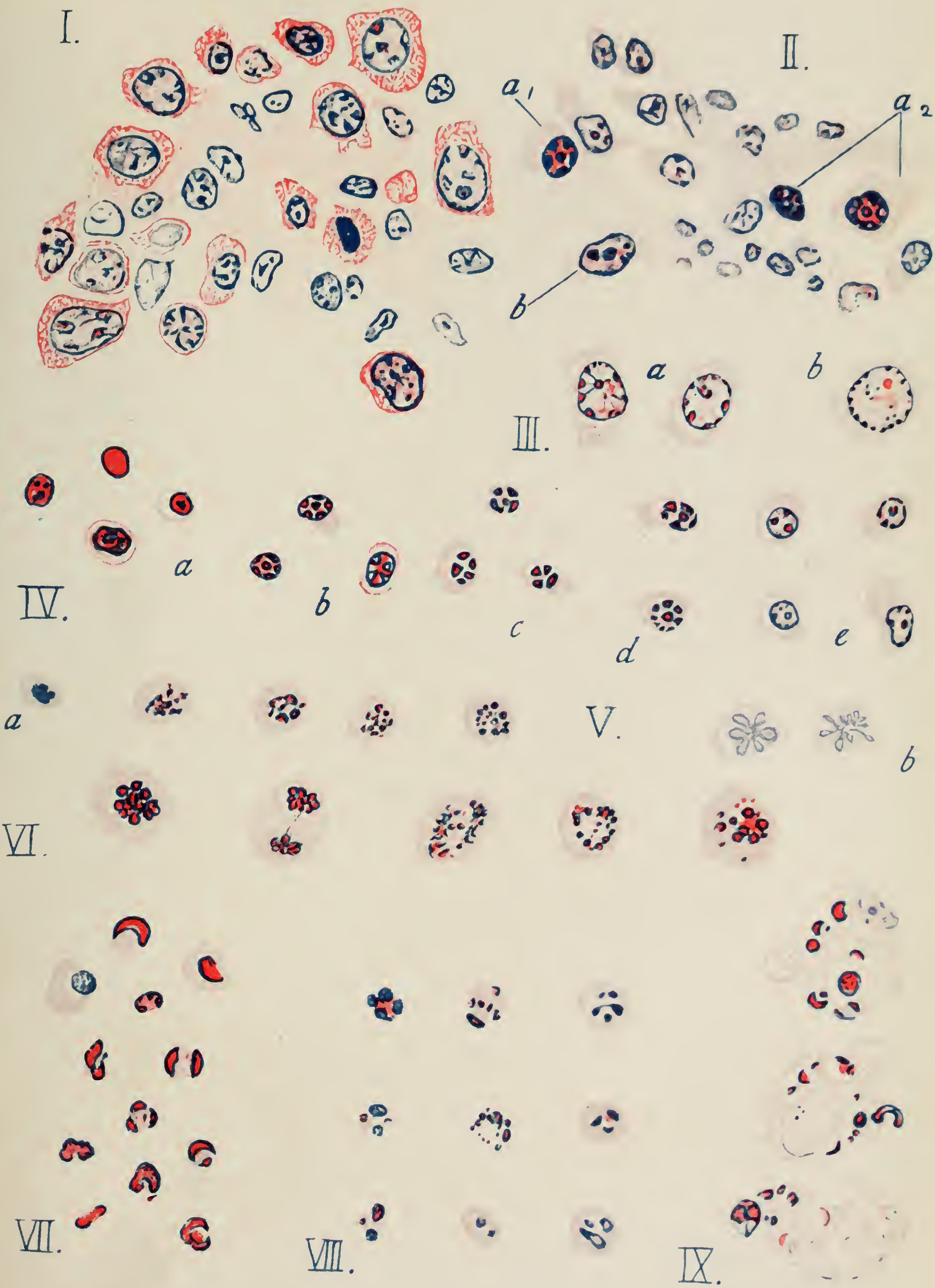
Fig. 6.—Various forms of karyorrhexis in large lymphocytes.

Fig. 7.—Hyperchromatosis and pyknosis of large and small lymphocytes.

Fig. 8.—Disintegration of compact homogenous nucleus of lymphocytes.

Fig. 9.—Pyknotic nuclei taken up by large phagocytes.

PLATE 1



ON THE OCCURRENCE OF HEMOLYTIC STREPTOCOCCI IN THE STOOLS OF SCARLET FEVER

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This work is one step in a study of the mechanism of infection from the gastro-intestinal tract. Most of the bacteria swallowed are believed to be killed in the acid stomach contents but it is possible that pathogenic bacteria may reach the intestines enmeshed in food particles or washed through with water. In order to establish the relationship of the bacteria in the intestines to foci of infection it is first necessary to know whether pathogenic bacteria are present in the intestines, how frequently, and in how great numbers. Hemolytic streptococci have been chosen because of their frequent occurrence in the mouth, ease of identification, and possible relationship to certain infectious processes. As hemolytic streptococci occur in great numbers in the throats of scarlet fever patients, a systematic study of the stools in scarlet fever was undertaken to determine the presence or absence of these organisms.

There is little in the literature with regard to studies of hemolytic streptococci in the feces. Broadhurst [1] isolated 9 strains of S. infrequens from 31 stools; Holman [2] reports 9 strains of S. infrequens Broadhurst and 4 strains of S. pyogenes, a total of 13 of 53. Oppenheim [3] found hemolytic streptococci in 5 stools from 15 normal individuals; and D. J. Davis [4] was unable to isolate any from the stools of 53 patients, 4 of whom had scarlet fever. Baermann and Eckersdorff [5] in a study of dysentery stools found streptococci which on blood agar were said to be definitely hemolytic. Winslow and Palmer,[6] Fuller and Armstrong,[7] and others have studied the fermentation reactions of fecal streptococci but they make no mention of growth of these organisms on blood agar.

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[1] Jour. Infect. Dis., 1915, 17, p. 277.
[2] Jour. Med. Research, 1916, 34, p. 377.
[3] Jour. Infect. Dis., 1920, 26, p. 117.
[4] Jour. Infect. Dis., 1920, 26, p. 171.
[5] München. med. Wchnschr., 1909, 56, p. 1169.
[6] Jour. Infect. Dis., 1910, 7, p. 1.
[7] Jour. Infect. Dis., 1913, 13, p. 442.

METHODS

Specimens were obtained from scarlet fever patients in the Durand Hospital. A portion of the stool was placed in a small sterile jar and examined from 1 to 48 hours later. When possible the specimen for examination was taken from the inside of the stool, and placed in about 3 c c of normal salt solution, making a moderately dense suspension; 2 or 3 loopfuls of this suspension were placed in another tube of salt solution, mixed, and with a small pipet about 0.25 c c was transferred to a third tube, mixed, and a similar amount to a fourth, making 3 dilutions. It was estimated that, using a wire loop 4 mm. in diameter, from 40 to 150 mg. of a soft-formed stool were used in making the first suspension. Dilutions were made with a 2 mm. loop. The water content of the stool is so variable that an exact estimate of the amount of feces obtained is impossible. Surface inoculations on plates were made, and also plates were poured, using 10% goat blood agar. After 24 hours' incubation the colonies with hemolysis were transplanted to blood or plain agar and microscopic examination made. Bile solubility, virulence, fermentation, agglutination and opsonic tests were made with various strains isolated from the feces.

Results Obtained from Repeated Stool Examinations from Individual Patients

	Number of Stools Examined	Number of Stools from Which Hemolytic Streptococci Were Isolated
1.	26	2
2.	22	4
3.	12	0
4.	10	1
5.	10	0
6.	9	3
7.	5	2

The total number of stools examined was 309, in 37 of which (12%) hemolytic streptococci were isolated. The stools were collected from 85 different patients, all with scarlet fever, and streptococci were isolated from 26 (30%). From 31 patients only one stool examination was made, of which 6 (19%) showed hemolytic streptococci. To 11 patients a cathartic was given and several stools obtained the same day. In 3 instances (18%) hemolytic streptococci were isolated.

The number of colonies of hemolytic streptococci on the plates on which they were noted was variable, but usually there were very few in comparison to the other organisms. On a blood-agar plate con-

taining some 200 or 300 colonies, usually 6 to 10 were definitely hemolytic streptococci. Occasionally on very thickly seeded plates with innumerable colonies only 3 or 4 hemolytic colonies were observed.

The age of the patients varied from 20 months to 45 years. Specimens of stools were examined at intervals of 1 to 62 days from the onset of the disease. In 2 instances hemolytic streptococci were isolated from watery stools, 13 from semisolid, 3 from soft formed, and 2 from hard; the greater number of stools examined were semisolid. There was no apparent relationship between the occurrence of hemolytic streptococci in the stools and the age of the patient, duration of the disease, or character of the stool. Stools obtained after the administration of a cathartic yielded a greater proportion of green-producing streptococci but the proportion of hemolytic streptococci was not increased.

On blood-agar surface streaked plates the hemolytic streptococci isolated grew, after 24 hours' incubation, as small, round, gray, somewhat raised colonies with a clear zone of hemolysis about 2 mm. in diameter, and a hazy border. The deep colonies under the microscope were small, biconvex or oval, with no red blood corpuscles visible for a diameter of 1 to 2 mm. On plain agar after 24 hours' incubation a small gram-positive coccus in chains was seen. All strains were insoluble in bile.

Fermentation tests were made with 22 strains of hemolytic streptococci isolated from the feces. These strains were grown 7 days in 1% lactose, mannite, salicin, and inulin; all fermented lactose and salicin but not mannite nor inulin, corresponding to Streptococcus pyogenes of Holman.[2]

Using 0.5 c c of an 18-hour broth culture, 20 strains were injected intraperitoneally into as many white mice. Of these, 13 died within 24 hours and hemolytic streptococci were isolated from the heart blood and peritoneal fluid; 4 of these strains, causing death of white mice in 24 hours were opsonified or agglutinated by the serum of a sheep injected with hemolytic streptococcus from scarlet fever.[8] One strain that killed a mouse was neither opsonified nor agglutinated by this serum and 2 other strains not killing mice were neither opsonified nor agglutinated.

Agglutination and opsonic tests were made using blood from a sheep injected with hemolytic streptococci isolated from the throat of

[8] Tunnicliff, R.: Jour. Am. Med. Assn., 74, p. 1386.

a scarlet fever patient before the appearance of the rash. In all, 11 strains were tested for opsonic and agglutinative reactions; of these, 6 strains were either agglutinated or opsonified by the immune serum. With two strains the points of opsonic extinction were 1 : 300 and 1 : 150, and each of the strains was agglutinated in a dilution of 1 : 200; 2 strains gave points of opsonic extinction of 1 :150 and 1 :90, but the determination of agglutination was unsatisfactory by reason of spontaneous agglutination in the normal sheep serum controls; 3 strains failed to give any opsonic or agglutinative reaction; 2 strains gave a positive opsonic reaction in a dilution of 1 :15, and 2 a negative reaction in the same dilution; these 4 strains were not agglutinated by the immune serum. It is of interest to note that one of the strains that did not react was isolated from a patient who had many hemolytic streptococci in the throat which also failed to react with the immune serum. There was some question clinically as to whether this patient had scarlet fever.

At intervals throat swabs from patients were streaked on blood agar plates and hemolytic streptococci found at one time or another in all patients examined. These organisms on blood agar appeared exactly similar to those isolated from the stools, and fermented lactose and salicin, but not mannite.

Green-producing streptococci were frequently noted and some fermentation tests were made: 6 strains fermented lactose and salicin, 2 strains fermented lactose, salicin and mannite, Streptococcus mitis and fecalis, respectively. Colon bacilli were, of course, encountered in great numbers; a large proportion were definitely hemolytic, a fact which caused difficulty in their differentiation from streptococci. There was a greater similarity between the deep colonies of hemolytic streptococci and colon bacilli than between those on the surface.

SUMMARY

Typical hemolytic streptococci were isolated from the feces of 30% of 85 scarlet fever patients. They occurred in the feces at irregular intervals with no definite relation to the age of the patient, character of the stool, duration or intensity of the illness. The number of colonies was small in comparison to the number of other organisms, such as B. coli, staphylococci, and green-producing streptococci, but when it is considered that only a small part of one loopful of the stool was studied it seems probable that hemolytic streptococci occur

in the stools of scarlet fever patients perhaps more frequently than is indicated by these figures. Six of 11 strains of hemolytic streptococci isolated from the stools of scarlet fever patients were agglutinated or opsonified by immune serum from a sheep injected with hemolytic streptococci obtained from the throat of an early case of scarlet fever.[8]

[8] Tunnicliff, R.: Jour. Am. Med. Assn., 74, p. 1386.

FURTHER OBSERVATIONS ON VARIETIES OF STREPTOCOCCI WITH REFERENCE TO HEMOLYSIS

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The significance of whether strains of streptococci lake blood or not is of importance to the clinician from the standpoint of pathogenesis and to the bacteriologist from the standpoint of classification. The plate method has been the usual one used to determine whether or not strains of streptococci have hemolytic properties. Various types of hemolytic strains have been reported by different workers, especially by Smith and Brown.[1] They describe the alpha and beta types. Brown[2] has later reported another type of hemolytic zone on the blood-agar plate, the alpha prime type.

To standardize a method for general use the Medical Department of the United States Army[3] in 1918 adopted the method of using a definite quantity of washed rabbit corpuscles with a definite quantity of a young rich broth culture of the strain to be tested. If hemolysis occurred the organism was considered as belonging to the hemolytic group. This method is more cumbersome, takes more work, and requires a longer time to determine whether or not a strain is hemolytic and should be justified only on superiority over the plate method or any other method requiring less time and energy.

In the work reported an effort has been made to study the ability of strains of streptococci grown on artificial medium to produce lysis of red blood cells and in doing this work a comparison of the army method and the plating method, when either rabbit or sheep blood is used, was made. The degree of hemolysis produced at the end of 2 hours by the method recommended by the United States Army is compared with the plate method, judging the hemolysis on the plate by the size and the clearness of the zone produced in from 18 to 24 hours.

[1] Jour. Med. Research, 1915, 31, p. 455.

[2] Monograph of Rockefeller Institute for Medical Research, No. 9, 1919.

[3] Methods for the Isolation and Identification of Streptococcus hemolyticus Adopted by the Medical Department of the United States Army, New York, 1918.

Meat infusion agar with a reaction of 0.5% acid was used and to this 10% of defibrinated blood was added. One hundred and sixteen strains[4] from various sources were tested. Of these 116 strains, 15 were from the blood of patients postmortem, 16 from tonsils removed in the dispensary, 51 from normal throats, 9 from normal horse feces, 7 from throats of influenza patients, 2 from pneumonia sputums, one from acute pharyngitis, one from pus from a frontal sinusitis, and 16 from cultures of throats which showed positive findings for diphtheria. These cultures, all except those isolated from diphtheria throats, were cultures that had been kept on artificial medium, meat infusion blood agar, for 3 years and transferred at intervals of 2 months. The work reported in this paper was done with strains picked from old cultures which had not been transferred for 4 months.

COMPARISON OF ARMY METHOD WITH PLATING METHOD WHEN RABBIT BLOOD WAS USED

Of the 15 cultures isolated from pathogenic processes, 14 gave a ++++ degree of hemolysis according to the army method. All of these 15 strains showed a zone of hemolysis on the rabbit blood-agar plate with a diameter of from 1-4 mm. The degree of clearness of hemolysis, with the exception of a few scattered colonies on the plates, ranged from 3 to 4 plus. Strain 114 gave no hemolysis by the army method but on plating on the blood agar a zone of hemolysis of 1 mm. in diameter and a +++ degree of clearness of hemolysis was produced. This strain was tested at other determinations with similar findings.

All of the 16 cultures isolated from removed tonsils gave a ++++ hemolysis according to the army method, except one. This one strain (52) gave a ++ degree of hemolysis. All of these 16 strains gave a 3 to 4 plus degree of clearness of hemolysis on the blood agar plate with zones ranging in diameter from 1 to 4 mm.

Of the 51 strains from the normal throats, 38 gave ++++, 4 a ++, 2 a + degree of hemolysis by the army method, and 7 gave no hemolysis at all. On the blood agar plate all of these strains produced zones of hemolysis with a diameter of 0.5-3 mm. and a degree of clearness of 2 to 4 plus. All of the strains from the normal throat that gave no hemolysis by the army method produced zones of hemolysis on the rabbit blood-agar plate with diameters of 0.5 mm. and a high degree of clearness in the zone of hemolysis. Of the 38 strains giving a ++++ degree of hemolysis by the army method only 3 produced zones of hemolysis having an average diameter of less than 1 mm. On the other hand, the 7 failing to hemolyze by the army method, although producing small zones of hemolysis, produced a high degree of clearness in the small zone of hemolysis in the rabbit blood.

All of the 9 strains isolated from normal horse feces produced a ++++ degree of hemolysis according to the army method. All of these showed a zone of hemolysis on the blood agar plate with a diameter of 1-3 mm. and a 3 to 4 plus degree of clearness.

A ++++ degree of hemolysis was produced by the army method by all of the 11 strains from the throats of influenza patients (7), pneumonia sputum (2), acute pharyngitis (1), and a frontal sinusitis (1) with one exception, strain 413, which gave a + degree of hemolysis. All of these 11 strains

[4] Jour. Infect. Dis., 1920, 26, p. 93.

hemolyzed blood on the blood-agar plate with a zone of hemolysis of 1-2 mm. in diameter. None of them dropped below a +++ degree of clearness of hemolysis in the hemolyzed zone.

All of the 16 strains recently isolated from diphtheria throats gave a ++++ degree of hemolysis by the army method with 2 exceptions. One (513) of these gave a ++ degree of hemolysis and one (501) failed to hemolyze at all. On the blood-agar plate all of the 16 strains produced zones of hemolysis varying from a 3 to 4 plus degree of clearness, with the exception of 2 in which ++ degree of clearness was produced.

Comparison of Sheep Blood in Both Army and Plating Methods

In the army method the use of rabbit blood is recommended. A comparison of sheep blood with rabbit blood was made with these 116 strains. When sheep blood was used with the 15 strains from necropsies, all but one strain produced a ++++ degree of hemolysis by the army method. This strain, 114, as with the rabbit blood, failed to produce any hemolysis at all by the army method. On the blood-agar plates the diameters of the zones of hemolysis as on the rabbit blood plate ranged from 1-4 mm. The number of large zones and the degree of clearness on sheep blood was slightly higher than on rabbit blood.

Of the 16 strains from tonsils only one (52) failed to produce a ++++ degree of hemolysis by the army method with sheep blood. The same results were obtained with rabbit blood. The size of the colonies on the blood-agar plate and the degree of clearness of the hemolyzed zones was slightly higher with the sheep blood.

Of the 51 throat strains 39 gave a ++++ hemolysis with the army method when using sheep blood as compared with 38 when using rabbit blood. Three gave a ++ with sheep blood as compared with 4 when rabbit blood was used. Seven failed to hemolyze sheep blood as compared with 7 that failed to hemolyze rabbit blood. As in strains isolated from other sources, these throat strains produced hemolysis in the blood-agar plate with a little higher average diameter and a greater degree of clearness in the hemolyzed zone when sheep blood was used.

As when rabbit blood was used, all the strains isolated from normal horse feces hemolyzed sheep blood to a ++++ degree by the army method. The size of the average diameter and the degree of clearness on the blood-agar plate with sheep blood was slightly higher than when rabbit was used.

Nine of the 11 strains from throats of influenza patients, pneumonia, sputums, etc., produced a ++++ degree by the army method with both rabbit and sheep blood. The size of the hemolyzed zones and the degree of clearness was as usual slightly greater with sheep blood.

All of the 16 strains isolated from diphtheria throats gave a ++++ hemolysis by the army method except one, as compared with the rabbit blood with which 3 failed to give a ++++.

The comparison between rabbit and sheep blood when used with the army method may be summed up as follows: Of the 116 strains from various sources there were 10 failures to hemolyze by the army method when both rabbit and sheep blood were used, and the strains which failed to hemolyze were the same in both cases. This was

carefully checked. The 10 strains were grown in glucose blood broth for 2 weeks and retested with the same results. There was no complete failure to hemolyze on the blood-agar plate, although the size of the zones of hemolysis and the clearness of hemolysis in the zones varied. There is a slightly higher percentage of complete hemolysis with the army method when sheep blood is used than when rabbit blood is used in the ratio of 104 to 100.

PRODUCTION OF GREEN DISCOLORATION OF BLOOD BY HEMOLYTIC STREPTOCOCCI

Since Schotmüller's [5] work with the blood-agar plate to differentiate strains of streptococci the question of hemolysis and the production of green discoloration on the blood-agar plate has been studied from various angles. Butterfield and Peabody [6] found that pneumococci were able to convert oxyhemoglobin found in the red blood cells of the rabbit into methemoglobin. Cole [7] states that the green produced about the colony by pneumococci on the blood-agar plate is methemoglobin. He also found that the pneumococci produced a hemotoxin seemingly of endotoxin nature [8] and that this hematoxin would lake red blood cells. Most observers of the green-producing streptococci and pneumococci on the blood-agar plate have noted that at 18-24 hours a green zone appears around the colony and that if observed later partial hemolysis can frequently be seen. There is a peripheral zone of green about the zone of hemolysis.

While plating out the series of 116 hemolytic strains of streptococci to determine whether they had retained their power to bring about hemolysis of the sheep blood, I noticed a few green colonies scattered among the typical beta hemolytic colonies on the sheep blood-agar plates. This led me to make observations on all the strains. Of the 116 strains observed, 54 showed from one to several green colonies scattered among the typical beta hemolytic colonies on the sheep blood-agar plates. The following table shows the number of strains in each group which produced some green colonies. It also shows the average size of the hemolytic zones on the blood-agar plates of those strains capable of producing green colonies as compared with the remaining strains which did not show the presence of green colonies scattered among the hemolyzers on the sheep blood-agar plates.

[5] München. med. Wchnschr., 1903, 50, p. 849, 909.
[6] Jour. Exper. Med., 1913, 17, p. 587.
[7] Jour. Exper. Med., 1914, 20, p. 363.
[8] Jour. Exper. Med., 1914, 20, p. 346.

Results of Observations on All Strains

Source	Number of Strains	Strains with Green Colonies	Average Size of Hemolytic Zones of Strains Producing	
			Green Colonies Mm.	No Green Colonies Mm.
Necropsies	15	6	2.5	2.8
Removed tonsils	16	9	2.7	3.6
Normal throats	51	30	1.6	3.0
Influenza throats	7	2	2.0	2.7
Pneumonia, sputum, etc.	4	2	2.0	2.0
Normal horse feces	9	0	...	3.5
Diphtheria throats	16	5	1.2	3.0

It is noted that strains from all sources gave green colonies except those strains isolated from normal horse feces. All of these strains were strong hemolyzers by the army method. More than 50% of the strains from tonsils and normal throats were capable of producing a few typical green colonies on the blood-agar plate. It was noticed throughout the work, as shown in the table, that there is a relation between the size of the clear zone of a strain and its ability to produce green colonies. The table shows an average diameter of the hemolyzed zone of those strains that produced green colonies to be 2 mm. in diameter as compared with 2.94 mm. of the average diameter of those strains that did not produce green colonies. It was also found that when a green colony was picked and replated that the tendency was to produce a smaller zone of hemolysis than was produced by the hemolyzers on the first plate.

To determine the nature and constancy of the green colonies produced, several strains were tested, with the following results:

147 A.

Plate (a) plated from stock showed many hemolyzers and a few greens.

Plate (b) plated from hemolyzer on plate (a) showed all hemolyzers.

Plate (b') plated from green on plate (a) showed hemolyzers and 2 greens.

Plate (c) plated from a green on plate (b') showed hemolyzers and few greens.

Plate (d) plated from green on plate (c) showed hemolyzers and few greens.

Plate (e) plated from green on plate (d) showed hemolyzers and few greens.

Plate (f) plated from green on plate (e) showed hemolyzers.

Plate (g) plated from small hemolyzer on plate (f) showed hemolyzers and a few slightly green colonies.

Plate (h) plated from slightly green colony on plate (g) showed only hemolyzers.

Picked and replated small hemolyzers daily for 2 weeks. All colonies were hemolytic with one exception, an inactive colony. This colony remained inactive for 2 generations and then became hemolytic.

147 B.

Plate (a) plated from stock showed all hemolyzers.

Plate (b) plated from hemolyzer on plate (a) showed hemolyzers and greens.

Plate (c) plated from hemolyzer on plate (b) showed hemolyzers and 2 green colonies.

Plate (d) plated from a green on plate (b) showed only hemolyzers.

Plate (e) plated from hemolyzer on plate (c) showed hemolyzers and 2 green colonies.

Plate (f) plated from a green from plate (d) showed only hemolyzers.

508.
Plate (a) plated from stock showed hemolyzers and greens.
Plate (b) plated from a green on plate (a) showed hemolyzers and greens.
Plate (c) plated from a green on plate (b) showed hemolyzers and one green.
Plate (d) plated from green on plate (c) showed all hemolyzers.
Plate (e) plated from hemolyzer on plate (d) showed all hemolyzers.

513 A.
Plate (a) plated from stock showed hemolyzers and few greens.
Plate (b) plated from a green on plate (a) showed hemolyzers and 2 greens.
Plate (c) plated from a green on plate (b) showed all hemolyzers.
Plate (d) plated from a hemolyzer on plate (c) showed all hemolyzers.

513 B.
Plate (a) plated from stock showed hemolyzers and greens.
Plate (b) plated from a green plate (a) showed hemolyzers and greens. Picked green from plate (b) and continued to replate daily for 2 weeks when inactive colonies appeared. These colonies when grown in dextrose broth for 24 hours and replated on blood agar showed only hemolyzers.

149 A.
Plate (a) plated from stock showed all hemolyzers.
Plate (b) plated from a good hemolyzer from plate (a) showed hemolyzers, greens and inactives.
Plate (c) plated from a hemolyzer on plate (b) showed hemolyzers.
Plate (d) plated from a green on plate (b) showed hemolyzers and greens.
Plate (e) plated from an inactive on plate (b) showed inactives. One of these inactives was inoculated into dextrose broth for 2 transfers.
Plate (f) plated from dextrose broth tube showed greens and hemolyzers.
Plate (g) plated from a green on plate (f) showed small hemolyzers.
Plate (h) plated from a hemolyzer on plate (g) showed small hemolyzers and one green.
Plate (i) plated from the one green on plate (h) showed hemolyzers and one slightly green.
Plate (j) plated slightly green from plate (i) and continued to replate daily for 2 weeks. At the end of this time all of the colonies were hemolytic.

The results suggest the possibility that hemolyzers may produce methemoglobin. Possibly the reason we do not detect the green is because it is overmasked by the hemolysis about the colony.

Holman[9] reports that hemoglobin is in time changed to methemoglobin. Mann[10] states that oxyhemoglobin is so readily converted into methemoglobin that if it be kept without special precautionary measures part of it becomes changed into methemoglobin. Webster[11] says that it is formed by the spontaneous decomposition of blood. Blake[12] states that the hemolytic streptococci cause complete hemolysis within 10-30 minutes but that in this time no methemoglobin is produced. Cole,[7] in testing out various kinds of sugars to determine whether pneumococci cause the formation of methemoglobin only in the presence of glucose or that this sugar might be replaced by one with a different molecular configuration, found that methemoglobin was not produced by hemolytic streptococci. A method by which hemolysis could be stopped and at the same time the possibility of the production of methemoglobin not be interfered with was sought. Ruediger[13] found that the addition of glucose to blood agar hindered or retarded the hemolytic power of hemolytic strains.

It has been found that on glucose blood-agar plates no hemolysis occurs but that colonies appear having from a typical green color to a reddish brown color about the colony. When blood is added to agar

[9] Jour. Med. Research, 1916, 34, p. 377.
[10] Chemistry of Proteids, 1906, p. 491.
[11] Diagnostic Methods, 1916, p. 411.
[12] Jour. Med. Research, 1917, 36, p. 99.
[13] Jour. Infect. Dis., 1906, 3, p. 663.

containing no sodium chlorid, a transparent hemolyzed blood-agar plate results. Hemolyzers were grown on these plates. No signs of hemolysis could be noted and neither was there any visible indication by color on the blood-agar plate that methemoglobin was produced in 24 hours. After 48 hours a green color was noted about the colonies on the blood-agar plates. Blood was added to salt free plain broth. This gave a transparent hemolyzed fluid. This broth when inoculated with a hemolytic strain showed a greenish color in 24 hours. This blood broth culture was tested with the spectroscope after 24, 48, 72, and 96 hours of incubation at 37 C. and it was found that a typical methemoglobin spectrum was present. Hemolyzers were grown in salt blood broth and tested by the spectroscope and the typical methemoglobin band in the red of the spectrum was noted. In both of these conditions noninoculated controls were tested and gave no indications of the presence of methemoglobin either from gross appearance or by spectroscopic determination.

The method of hemolyzing blood by heat was tried. It was found that if 10% of defibrinated blood was added to salt agar at 80 C. and poured in a few seconds into a petri dish that the blood would show almost complete hemolysis. Such plates were inoculated with hemolyzers and incubated at 37 C. for 24 hours. Green-producing colonies appeared. Salt broth tubes, to which a similar amount of defibrinated blood was added when the broth was at 80 C. and immediately cooled, were inoculated with hemolyzers. After 24 hours a noticeable dark green color appeared in the medium and on spectroscopic examination the typical methemoglobin band in the red was present. Control uninoculated tubes were also tested with the spectroscope. These tubes showed the presence of methemoglobin. Blood was added to salt agar at boiling temperature and immediately plated. Fifty hemolyzers were stroked on the plates. In 24 hours typical green colonies were present. The intensity of the green increased when the plates were incubated for 48 hours. Nonhemolytic strains were also tested on this heated blood medium and produced colonies similar in all respects to those of the hemolyzers. Blood added to boiling salt broth was quickly cooled as in plating the agar. This blood broth was inoculated with hemolytic strains of streptococci and tested at 24, 48, and 72 hours of incubation at 37 C. The typical methemoglobin spectrum was found. Nonhemolytic streptococci were grown under similar conditions and tested with the same results. A noninoculated heated

blood broth tube was tested a few minutes after heating and no methemoglobin was detected. After incubating such a tube for 24 hours, methemoglobin could sometimes be detected.

Methemoglobin, according to the spectroscopic determination, appeared sometimes in uninoculated tubes of blood heated to either 80 C. or to boiling. Blood which had been added to sterile broth and had stood at room temperature for several weeks was tested with the spectroscope and the presence of methemoglobin was easily detected. It seems evident that the hemoglobin in the heated or unheated blood may on standing become changed to methemoglobin without bacterial action; but, considering the green on the heated or unheated blood-agar plate as an evidence of the presence of methemoglobin and the fact that unheated uninoculated red blood cells, hemolyzed or unhemolyzed, did not show indications of methemoglobin after 24 hours of incubation at 37 C. by the spectroscopic determination and that similar tubes inoculated with hemolytic streptococci did, it would seem that hemolytic strains may produce or may hasten the production of methemoglobin in the sheep red blood cells.

Cole [7] decided that the best explanation of the bacterial process of producing methemoglobin from oxyhemoglobin is that there is at first a reduction process and then an oxidation process. It has been found (Mathews) that by heating oxyhemoglobin slightly the oxyhemoglobin is reduced gradually more nearly to hemoglobin (reduced hemoglobin). It is possible that by heating the blood at boiling for a few seconds some oxyhemoglobin is reduced to reduced hemoglobin and then hemolytic strains take up the process and oxidize the reduced hemoglobin to methemoglobin. Part of the hemoglobin may also be decomposed to globin and hematin. Mathews states that hemoglobin begins to decompose into hematin and globin at 64 C. It was found that not all of the hemoglobin was decomposed when heated to 80 C. or to boiling for a few seconds for in both cases the hemoglobin spectrum could be detected by spectroscopic determination. Globin is rich in various amino acids and probably for this reason produces a medium which is suitable for the good growth of streptococci. This medium has been found to be a good medium on which to grow Pfeiffer's bacillus.

The possibility that this green appearing on the heated blood-agar plate is sulphur-methemoglobin was considered. Sulphur-methemoglobin is produced by the union of hydrogen sulphid with hemoglobin

It is noticeable over the abdomen of persons brought to the necropsy table. This condition is known as pseudomelanosis. To a culture of hemolytic streptococci in blood broth in which typical methemoglobin bands were shown in the spectrum a few drops of dilute ammonium sulphid were added when the band immediately disappeared. This would indicate that the green produced by the hemolytic streptococci is methemoglobin and not sulphur-methemoglobin.

The fact that hemolytic streptococci may produce methemoglobin could account for strains described as the alpha type by Smith and Brown [1] in which the hemotoxin-producing power of the strain is weak and less hemolysis is produced and a slight amount of green is seen about the colony and the periphery of the hemolyzed zone. This fact could also explain Rosenow's observation of green colonies appearing among the hemolytic colonies and of my findings where strains which had been kept on artificial medium for more than 3 years were found which produced green colonies.

CONCLUSIONS

Hemolytic strains of streptococci when kept on suitable medium may retain the hemolytic property for at least 3 years.

The method recommended by the Medical Department of the United States Army does not seem to have any advantage over the plating method on the blood-agar plate in determining the degree of hemolysis. It seems possible to miss hemolytic strains by the army method when they can be detected by the plating method.

Sheep blood while it lakes slightly more readily than rabbit blood can be used with equal efficiency in either the recommended army method or the plating method.

Typical hemolytic strains after being grown on artificial medium from 6 months to 3 years may produce colonies which show a green color about the colony similar in all appearances to the colonies of Streptococcus viridans.

The appearance of these green colonies tends to be associated with a weakening in the ability of strains to hemolyze as is shown by the degree of hemolysis produced by the army method and the smallness of the size of the hemolyzed zone about the colony on the blood-agar plate.

All of the 50 hemolyzers tested produced a green discoloration of the sheep red cells on heated blood similar in all respects to the green produced by nonhemolyzers.

This green colored substance seems to be methemoglobin when compared with the green produced on the blood-agar plate by Streptococcus viridans and according to the spectroscopic test.

Methemoglobin is more readily produced by hemolytic strains of streptococci in heated blood than in nonheated blood.

PLAGUE-LIKE ORGANISMS IN THE WILD RATS OF SAO PAULO, BRAZIL

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During the study of the prevalence of Leptospira hemorraghiae in the wild rats of the city of São Paulo, a group of interesting organisms were encountered which were very puzzling at first in that they were so similar to the plague bacillus.

It was necessary to catch normal appearing wild rats in various parts of the city, choloroform them, macerate the kidneys in a sterile mortar, with careful aseptic precautions, and inject the resulting emulsion into the peritoneal cavity of guinea-pigs. The work went well up to a certain period, when we began to examine rats that had been caught in the chicken yard of Santa Casa, the large general hospital. One of every three or four of the guinea-pigs that were injected with kidneys of rats caught in this location died within 12 to 24 hours after they were inoculated, though the rats used in the experiments had all appeared normal. The dead guinea-pigs presented all the typical characteristics of animals inoculated with the bacillus of bubonic plague.

This finding was rather alarming, for though there were no known cases of plague in the city, the rats came from the grounds of a hospital at which it was strongly suspected that a recent death had resulted from plague, and the port of Santos, only 40 miles away, was suffering at this time with an outbreak of the disease. Our alarm was not quieted by subsequent study.

Smears from the peritoneal cavity of the dead guinea-pigs showed myriads of gram-negative, bipolar rods, with rounded ends, their morphology being characteristic of the plague bacillus. Pure cultures of the organism were readily obtained from the heart blood of the guinea-pigs.

It was obvious that we were working with an organism which, if it was not the plague bacillus, was certainly closely related to it, and we therefore made an immediate differential study of the organism

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Morphology.—The organism in fresh culture, or when examined directly from animal tissues, was a bipolar bacillus with rounded ends. It was non-motile, had no capsule and did not form spores. It stained readily with all the common anilin dyes, and was gram-negative. In a 3% sodium chlorid broth it formed involution forms. There was no chain formation, though in broth the bacilli were sometimes seen in twos and fours.

Agar.—The organism grew slowly on agar slants as whitish tiny transparent colonies. At the end of 24 hours the growth was barely visible. The luxuriance of the growth was greatly increased by the addition of a few drops of rabbit blood agar. It grew best at 37 C.; growth took place slowly at 20 C. also, but this is a much less suitable temperature for rapid development than 37 C.

Broth.—The growth was scanty and diffuse even after two weeks' incubation. Good stalactite formation was never obtained in broth cultures though small pellicles formed on the surface of the flask, which had been covered with oil, and extended from 1 to 2 c c into the medium, when the inoculated flasks were placed in a quiet solid place, and allowed to stand for four weeks.

Gelatin.—Growth took place slowly without liquefaction; a faint growth along the needle track was seen only after several days' incubation.

Litmus milk.—There was a faint change in the color of the litmus milk, due possibly to the fermentation of the very small amount, 0.08%, of dextrose-like sugar in milk. There was no coagulation. .

Potato.—The growth on potato was invisible. Examination of material from the surface one week after inoculation seemed to indicate that there had been some slight growth.

Hiss' Semisolid Medium.—There was slight acid formation without gas in dextrose, maltose, saccharose and mannite mediums. There was no acid formation in lactose or dextrin. These carbohydrates were the only ones available and the purity of saccharose was not above question.

Sugar Broth Tubes.—Sugar broth tubes were made and the hydrogen-ion concentration estimated seven days after inoculation. The sugar broth tubes all showed a scanty growth. The same carbohydrates were fermented as in the semisolid mediums. The acid-end point for all tubes in which there was fermentation was about 6.3—estimated by the colorimetric method. The sugar tubes in which no fermentation occurred and also the control uninoculated tubes gave a hydrogen-ion of 7.6.

MacConkey's Sodium Taurocholate Neutral Red Agar.—Repeated inoculations of MacConkey's medium gave negative results. The use of this medium is one of the best methods of differentiation between the plague bacillus and the pasteurellosis group, according to the Indian Plague Commission,[1] because the plague bacillus grows readily on this medium, whereas the other members of the hemorrhagic septicemia group do not.

It is clear therefore that up to this point the chief differential characteristic between the true plague bacillus and the organism we were studying, was that our strain did not grow on MacConkey's medium, and characteristic stalactite formation in broth had not been

[1] Jour. Hyg., 1908, 8, p. 302.

obtained. This evidence was entirely negative and under the circumstances was far from sufficient. Further confirmation of the cultural findings were urgently needed.

Inoculation in Guinea-Pigs.—A very small quantity, 0.005 c c of a 24-hour scanty broth culture inoculated intraperitoneally into a 300 gram guinea-pig always produced death in 14 to 20 hours. There was a wide area of edema and hemorrhage about the point of inoculation, the inguinal and auxiliary nodes were enlarged, deep red in color, with marked hemorrhagic injection about them. The abdomen contained an excess of clear or slightly cloudy serous fluid, with an occasional bit of flaky white fibrin on the surface of the liver. The abdominal vessels were deeply congested. The spleen was enlarged. The pleural cavity contained as a rule 10 to 20 c c of a clear pleural fluid and the lungs were deeply congested. The abdominal and pleural fluid, the liver, and heart blood always contained large numbers of the typical bipolar organism. When the bacilli were inoculated subcutaneously into guinea-pigs in doses of 0.1 to 0.5 c c of a 24-hour broth culture the same pathologic picture was produced as by intraperitoneal inoculation, for a rapid and fatal septicemia ensued within a few hours. A different picture was seen when small doses, 0.005 c c to 0.01 c c, of a 24-hour broth culture were injected subcutaneously, for in these animals an abscess formation usually occurred at the point of inoculation. The animal died on the fifth to seventh day, with extensive fibrinopurulent peritonitis, pleuritis, and pericarditis. In some instances the heart was buried in a coat of fibrin 4 mm. thick. The bipolar organism was found in all the serous cavities and in the heart's blood in considerable numbers, though much less frequently than in the material taken from animals inoculated intraperitoneally.

Broth cultures of the bacillus were rubbed vigorously into the skin of the shaved surface of the abdomen of guinea-pigs, or scratched into the surface of the skin, but in only one instance was infection produced—a slight abscess formation at the point of the scratch, which did not result fatally. Cultures were also rubbed on the nasal mucous membranes, but we were never able to infect by this route. One of the best differential characteristics of the plague bacillus, according to Fritsche,[2] is that infection can be produced in guinea-pigs with the true plague bacillus by simply rubbing the broth culture into the shaved skin of the abdomen, whereas cultures of other plague-like organisms will produce no disease by this method of inoculation.

Rabbits.—The bacillus was very virulent for rabbits—0.005 c c injected intravenously killed an adult rabbit within a few hours (8 to 10) producing all the characteristics of a hemorrhagic septicemia. Intraperitoneal injections of 0.01 c c were rapidly fatal, as was 0.1 c c when injected subcutaneously. The bacilli were always recovered from the heart blood.

White Rats.—White rats were much less susceptible to the bacillus, 0.1 c c of a 24-hour broth culture inoculated intraperitoneally into a half grown white rat, a dose large enough to kill 20 guinea-pigs, produced only moderate symptoms of illness. One half c c of a 24-hour broth culture inoculated subcutaneously did not kill, though a local abscess was formed.

Pigeons.—One of the strains proved pathogenic for pigeons, 0.5 c c of a 24-hour broth culture inoculated intrapectorally producing death in a few hours,

[2] Arb. a.d. Kais. Gesundheitsamte, 1902, 18.

and the heart blood was found swarming with bipolar bacilli. This is another differential characteristic of value, for the plague bacillus is never pathogenic to pigeons. This finding does not prove that the organism belonged to the fowl pasteurellosis, for some of the other members of the group are fatal to pigeons.

Toxin.—The organism was inoculated into 250 c c of plain broth, to which a few drops of rabbit blood was added, and kept at room temperature for 5 weeks. At the end of this time the broth was passed through a Berkefeld filter and the filtrate tested for sterility. Two or three c c of this filtrate contained sufficient toxin to kill a guinea-pig in 24 hours, when inoculated intraperitoneally. This production of toxin is not a differentiating characteristic, for not only the plague bacillus but other members of the hemorrhagic septicemia group also produced a soluble toxin.

Plague Serum.—A small amount of a 24-hour broth culture, 0.05 c c, was mixed with 2 c c of antiplague serum and the mixture injected intraperitoneally. A control animal was injected with the culture only. The animals died 18 to 20 hours later, the control animal succumbing within an hour of the animal inoculated with the mixture of bacteria and serum. This is an additional proof that the organisms were not true plague for the antiplague serum always gives some protection against the plague bacillus.

Agglutination.—Animals were inoculated first with killed and later with living cultures of the organism. Immunity was difficult to produce, and many animals were killed before a serum was obtained which agglutinated the specific strain.

Agglutination for the specific strain occurred in a dilution of 1:10 to 1:20 but no cross agglutination was obtained with any of the strains. Plague serum of a high immunizing titer obtained from the Butantan laboratories did not agglutinate any of the strains.

DISCUSSION

We lost in all six or seven guinea pigs which died from peritoneal infection following inoculation with the kidneys of normal appearing rats which were all captured in the same location. From these guinea-pigs three separate strains of plague-like bacilli were obtained. All three strains were found to be practically identical in regard to their morphologic and cultural characteristics and their virulence to animals, though one strain was virulent to pigeons, while the others were not, and no cross agglutinations were obtained.

It is clear from all the evidence, morphologic, cultural and the results of animal experiments, that we were working with an organism that belonged to the hemorrhagic septicemia group. The only evidence against this was the apparent fermentation of saccharose. We must remember, however, that the hemorrhagic septicemia group consists of a number of organisms, all very similar in morphologic and cultural characteristics and that the plague bacillus is simply the member of

this group that produces epidemics of disease in man and rats. The differentiation of the other members of the group from the plague bacillus is usually of more or less academic importance, for the other organisms encountered are usually isolated from frank epidemics in goats, sheep, fowls, horses, hogs, dogs, rabbits, etc., as the case may be.

We had before us a different and intensely practical problem, from the public health point of view. We accidentally found that, in a large city only 40 miles from a port that was at the moment known to be infected with plague, and on the grounds of a hospital where a suspected case of plague had recently come to necropsy, the rats harbored an organism whose morphology was exactly that of the plague bacillus. It is obvious that the burden of proof rested on us to determine accurately and quickly whether or not the organism was the plague bacillus, so that if the results were positive, immediate preventive measures could be taken against an outbreak of plague in man. If we could prove that it was not the plague bacillus but one of the other members of the hemorrhagic septicemia group, then the practical part of the problem was solved. (Before this paper was completed an explosive epidemic of plague did break out in another part of the city).

Let su marshal our evidence against the bacillus being the plague bacillus.

There was nothing about the morphology and staining characteristics that were inconsistent with its being the plague bacillus. In fact, every morphologic requirement for plague was fulfilled.

All the members of the hemorrhagic septicemia group may form stalactites under favorable conditions, so that this is not a good differential characteristic. We were unable to obtain good stalactites even after 5 weeks of growth but this may have been due to unsuitable conditions.

The failure of the organism to grow on maltose sodium taurocholate neutral red medium, though it is negative evidence, is of considerable differential diagnostic importance, since the true plague bacillus grows readily on this medium. The "Indian Plague Commission" considers this medium a valuable differential indicator. It is an item of evidence that needs confirmation, however.

In the first place, it is important to note that all the original rats examined were normal in appearance. This is unusual, to say the

least, if the organisms were the plague bacilli, though bacilli of low virulence for rats have been described. Since the organism did not kill white rats even when 0.5 c c of a 24-hour broth culture was inoculated, we must conclude that if the organism was the plague bacillus it had a very low virulence for rats. The pathologic picture produced by intraperitoneal inoculation of small amounts of culture, 0.005 c c, or large amounts subcutaneously, 0.1 c c, into guinea-pigs was characteristic of plague, but the picture produced by the inoculation of small amounts of culture, 0.005 c c subcutaneously into guinea-pigs was quite different from the usual typical picture in plague infected guinea-pigs.

More important evidence was the fact that repeated attempts to infect guinea-pigs by rubbing undiluted 24-hour culture into the shaved skin of the abdomen or into the membranes of the nose were unsuccessful in producing the disease. Fritsche says that true plague will always be produced in guinea-pigs by this method and considers it one of the best differential diagnostic signs. One of the strains proved pathogenic for pigeons, a characteristic that is not found in the true plague bacillus.

From this evidence, therefore, we may reasonably conclude that the organism isolated from the kidneys of normal appearing São Paulo rats belonged to the hemorrhagic septicemia group, but were not plague bacilli. To what group they belonged—fowl, horse, goat, sheep, dog, or rabbit—is more difficult to say, and all are possible since the rats were in intimate contact with all these animals in the Santa Casa yard. The important question in which we were interested was whether or not the disease was plague, and we were able to arrive at a definite conclusion on this point.

Bacillus Enteritidis.—One other organism was encountered in São Paulo rats that resembled the plague bacillus somewhat in its morphologic characteristics. The guinea-pig, which was inoculated with the kidneys of rat 15, died of general peritonitis during the 34th hour. Microscopical examination of the peritoneal fluid showed a pure culture of an organism which was gram-negative and stained bipolarly with methylene blue. Cultures from the heart blood were made on agar slants and incubated. The following morning the agar slant was covered with a diffuse moist whitish growth. This characteristic of rapid growth was alone almost sufficient to rule out the possibility that the organism was the plague bacillus. Subsequent study rapidly classified the organism as belonging to the B. enteritidis group. Bates[3] found a similar "safety pin"

[3] Proc. Canal Zone Med. Assn., 1916, 7, p. 61.

organism in an enlarged gland of a rat examined at necropsy in the Canal Zone, which he identified as belonging to B. enteritidis group, and numerous epizootics have been recorded in rats and rabbits, due to the gaertner bacillus. According to some authors, the gaertner bacillus is a normal inhabitant of the rat's intestine. It is one of the easiest bipolar organisms to differentiate from the plague bacillus because of its rapid growth on all ordinary mediums, with brownish growth on potato, and its motility.

CONCLUSIONS

Three strains of bacilli were isolated from normal appearing rats of São Paulo, at a time when the city was in close proximity to and in constant danger from bubonic plague. It was proved by cultural methods and animal inoculation that these organisms were not plague but belonged to the closely allied pasteurellosis group.

A rapid and sure diagnosis of B. pestis is not always a simple matter. One is not justified in relying on one or two modes of differentiation only, but one must apply every differential method known.

B. enteritidis, which may resemble B. pestis somewhat in morphologic and staining characteristics, was isolated from the kidneys of a normal appearing rat. The differentiation between B. pestis and B. enteritidis is a simple and rapid process.

ETIOLOGY OF ACUTE GANGRENOUS INFECTIONS OF ANIMALS: A DISCUSSION OF BLACKLEG, BRAXY, MALIGNANT EDEMA AND WHALE SEPTICEMIA

STUDIES ON PATHOGENIC ANAEROBES. I *

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INTRODUCTION

A piece of work on which I have been engaged, concerning the tissue-invading anaerobes, involved the collection of as many strains of such organisms as possible. At the suggestion of Dr. Karl F. Meyer, a fairly large number of strains of animal origin were collected and analyzed. It was thought that, besides being in itself of economic importance, the investigation of such a collection would be of assistance in the study of organisms causative of gas gangrene in man, for which branch of research the available strains of human origin were found inadequate. As these animal strains in turn threw new light on the subject of the infections of animals, it was thought appropriate to present under the above title certain data in connection with them. It also seemed desirable to make a rather extensive critical review of the literature of anaerobic animal infections.[1]

There are many obscure points concerning the anaerobic invasions of animals. Human wound infections with their resulting cases of gas-gangrene have been studied extensively during the period of the war and they are found to be varied in their etiology and frequently polyspecific in their nature. The anaerobic organisms causative of human gas gangrene are usually placed in what I believe to be three important groups, which are considered by some workers (Weinberg and Séguin (b), the Anaerobe Committee) to be three species, namely, Vibrion septique Pasteur (the bacillus of Ghon and Sachs); B. oedematiens Weinberg and Séguin (closely related to but not identical with B. oedematis maligni II of Novy); and B. welchii (B. phleg-

* This work was commenced during the author's tenure of the Alice Freeman Palmer Fellowship of Wellesley College.

[1] On account of the length of this paper it was found necessary to omit from the Journal a large portion of the literary review which was included in Parts I and III. This portion is included in the author's reprints and in the "Collected Reprints of the Hooper Foundation," volume 5, 1919-1920.

mones emphysematoseae Fraenkel, B. perfringens Veillon and Zuber). Rarely other invading organisms are found which are not to be identified with the above. I cite B. histolyticus, B. fallax and B. aerofoetidus Weinberg and Séguin, B. egens Stoddard, and have myself found a few such organisms that appear to be new. In the war literature there seem to be a host of faulty identifications, of associations of unrelated organisms, and of descriptions of mixed cultures. I shall confine myself in this paper to a discussion of the infections of animals.[2]

I. A General Discussion of the Principal Organisms Involved in the Anaerobic Infections of Domestic Animals

The differentiation of the disease called blackleg from that which is usually called malignant edema has been undertaken in all modern textbooks of veterinary pathology. The criteria used for such differentiation are usually seriously open to criticism, and in some cases are the cause of decades of misunderstanding of the subject. So serious have these misconceptions become that vibrion septique strains have been used as standards by which to identify B. chauvoei. I cannot otherwise explain the statements of M. Nicolle, Cesari, and Raphael, who correlate the two organisms. The fact that the Pasteur Institute sent this laboratory a strain of the vibrion septique group, which was known as one of LeClainche and Vallée's classic blackleg strains, lends color to the suggestion that Nicolle, Cesari, and Raphael did not work with true strains of B. chauvoei. Weinberg and Séguin come to the conclusion, through a review of the literature, that vibrion septique and B. chauvoei are identical (b). I find it impossible to confound the organisms of these two types if they are in pure culture and if adequate facilities are at hand for their study.

It may be broadly stated that the anaerobic invaders of mammalian tissue form a large group, the members of which are not truly parasitic

[2] The bibliography of these diseases is sometimes difficult to quote in an orthodox fashion. Various authors of textbooks give long lists of articles consulted by them, and frequently in the account which follows such a bibliography no indication is given as to which of the many authors is responsible for the statements made in the text. A large number of references in early volumes of veterinary journals are difficult of access. One textbook gives an excellent account of braxy with no bibliography at all, while other important textbooks give references to a few but not to all of their authorities. In general, I have tried, in referring to an authority who quotes from another, to give the quoted statements verbatim; for the sake of clearness I have inserted the page number with the name of the author in the text. A complete alphabetical list of references will be found at the end of the paper. The letter "p" before a number refers to page numbers in this paper.

but may become so under diverse conditions. The species of the invaders are many, and they may be organized into groups which may best be likened to genera. Chief among these groups in incidence in animal infections is the vibrion septique group, which has a wide range of pathogenicity among animals. Second in incidence is the blackleg group, which has a narrower range of pathogenicity among domestic animals. Oedematiens group and Welch group infections may occur in animals, but they are comparatively rare. One should keep in mind the possibility of the occurrence of infection by organisms of other groups, and the question of invasion initiated by proteolytic organisms is to my mind entirely open. We are only on the threshold of the study of the anaerobic tissue invaders. Careful investigation of a large number of strains is necessary before definite generalizations concerning them are warranted. As far as my investigations go, I am prepared to define with some accuracy the principal groups of anaerobic invaders. Later researches may compel me to change somewhat the details of these definitions. Such definitions are here given for the vibrion septique and blackleg groups only.

Recently a bacteriologist declared to me in a private communication that a certain strain corresponded to the descriptions of the bacillus of blackleg which were "found in the literature." The culture proved to contain several anaerobic organisms of various types. On thinking the matter over in the light of the reading that I had been doing I could not blame him for his diagnosis. Perhaps this paper will serve to stimulate a more exact study of a sadly misunderstood group of bacteria. It should be said, and with emphasis, that the careful study of the organisms involved is of infinitely more value than any unstandardized pathologic observations or any epidemiologic ones. In many cases in which reports are given concerning blackleg infections or malignant edema infections it is impossible to tell which type of organism was present, whether both types were present, or whether an organism of another group may not have been present. No one is qualified to make a statement as to the etiologic factor in an anaerobic infection without bacteriologic investigation, and the literature contains so many confusing statements as to the bacterial entities involved that no one but the fortunate bacteriologist who has several strains to study and the time to study them is qualified to determine even the group affinities with certainty. When, however, the nature of the

groups is understood, and when a technic is developed for their differentiation, one does not understand how the two could ever have been confused. They stand clear and separate as night and day, and mixtures of the two are quickly recognized.

1. CRITERIA OF DIFFERENTIATION EVOLVED DURING THE PRESENT RESEARCH

To save much confusion I shall here insert a definite statement as to the behavior in my hands of the two chief types of anaerobic animal invaders. I do this with due apology to others [3] who have adequately described these types, in order that the reader may understand definitely what my criteria of distinction are. I base my group identifications of the vibrion septique type on two strains of this type (VS and MP) sent to Miss Robertson and to Dr. Meyer by the Pasteur Institute. They are not specifically identical but are both referable to the group as I conceive it. Both are supposed to have been strains of Pasteur's. The blackleg group identification with B. chauvoei is based on the description of Arloing, Cornevin, and Thomas, see reprint.

(a) The Blackleg Group.—I should define the blackleg group [4] as follows:

1. Pathogenic Characters: Organisms producing a deeply hemorrhagic inflammation and necrosis in the muscles of the guinea-pig, with some sanguinolent infiltration, with little or no gas production, little interstitial regional edema, and only local injection of the peritoneum and rarely any injection of the small intestines. In guinea-pigs, dying of blackleg infection, bile frequently enters the small intestines in large quantities; the gallbladder is usually empty, the intestines do not contain an unusual amount of gas; the peritoneal surfaces are not excessively moist. The blackleg organisms are not highly pathogenic for laboratory animals when compared with the organisms of the vibrion septique group; some strains are in my hands pathogenic for rabbits.

2. Morphologic and Staining Characters: Bacilli that are usually gram-negative (carbol methyl violet Gram stain, see p. 401, b). In the vegetative form, in animal tissue and in meat medium, they usually stain uniformly and palely. In the orgont [5] form and in the sporulating form they may or may not stain clearly, depending on the amount of carbohydrate in the

[3] Von Hibler (a and c) has a clear understanding of both groups. Markoff, Wulff, and several other workers have a definite understanding of blackleg. Ghon and Sachs, Weinberg and Seguin (b), and Robertson (a) have adequately described the vibrion septique type. Meyer (a and c) and McIntosh give the salient characters of both types, and lastly both types are described by the Medical Research Committee (Special Report No. 39).

[4] A detailed classification of the species that are represented in my collection and make up these groups, as well as certain related groups, is in process of elaboration.

medium. In case they stain clearly they stain less intensely than do vegetative forms; in case the protoplasm is granular, the granules may stain strongly, taking the Gram stain; the remaining substrate is pale and usually gram-negative. The vegetative forms are comparatively slender, usually averaging in a 24-hour meat culture at least four times as long as wide. Actual measurements vary greatly. The vegetative and sporulating forms of most strains show little tendency to form filaments. I have never seen strains of this group which form filaments on the liver of the guinea-pig. The orgont forms are often far larger than the vegetative forms. They are extremely polymorphic, varying greatly within the group. Some are rods, some are rounded or pointed oval forms, some are lemon shaped, some are almost spherical. Spores form early in the development of a culture—in meat medium after approximately 20 hours' growth, and sporulating rods may usually be found at the site of inoculation in the guinea-pig. Spores are always oval, never spherical; they may vary greatly in their proportions and size in one strain, sometimes being long with parallel sides; they are usually situated subterminally in the bacillus (though terminal and median spores occur) and they frequently lie sidewise in the bacillus. This character is more usual for some strains than for others; it is not characteristic of blackleg alone. Rods containing two spores are occasionally seen.

3. Cultural Characters: Strictly anaerobic organisms generally of shy growth habit, which show no proteolytic action on meat, serum, or egg mediums, but which liquefy gelatin. They do not in my hands grow on fresh brom-cresol milk alone but grow well even in old milk with blood in it, forming in 2-4 days a soft clot which is torn by ascending gas and is usually fragmentable by shaking. They split glucose readily, producing gas in meat medium and turning it pink. They are strictly nonproteolytic in the anaerobist's sense of the term. They are violently hemolytic; they autoagglutinate with extreme readiness; they are flagellate and motile (see p. 401, a).

Their deep colonies in liver agar are small, rather slow to develop, fundamentally lenticular in structure and are subject to variations according to species. The colonies rarely show tufted projections and do so only after two or three days' incubation. These projections are not loosely woolly but are clumpy and downy.

(b) The Vibrion Septique Group.—I should define the Vibrion septique group as follows:

1. Pathogenic Characters: Organisms producing a moderately marked serohemorrhagic inflammation of the muscles of the guinea-pig, marked interstitial infiltration, a moderate degree of serous subfacial regional edema and usually a large amount of gas; and producing almost invariably extensive

[5] Orgont, participial s em cf ὀργάω 'swell', properly 'swell and teem with moisture', used, for example, of fruit swelling as it ripens. Compare schizont from σχίζω 'split.' I would suggest orgont as an adjective descriptive of the organisms that have lost their bacillary form or size and have swollen, presumably preparatory to sporulation. "Orgont form" may be taken to include all swollen forms in which spores are not visible, whether the fate of such an individual is sporulation or dissolution or even division. "Orgont" may be used as a noun, like schizont. Rods which do not swell preparatory to sporulation may be said to sporulate without "orgont formation." Orgonts may or may not show protoplasmic disorganization.

injection of the serous membranes and of the intestines. In guinea-pigs dying of a vibrion septique infection the bile is freely discharged into the small intestines in large quantity, the gallbladder is usually empty, and the intestines contain a large amount of gas. The peritoneal surfaces are excessively moist.

2. Morphologic and Staining Characters: Organisms usually gram-positive (carbol methyl violet Gram stain, see p. 401, b), though not intensely so, as are the organisms of Welch and Bifermentans type. In the vegetative form in animals and in meat medium they stain uniformly and rather deeply with anilin dyes. In the orgont form, and frequently in the sporulating form, they stain irregularly. In the animal body a localization of deeply staining protoplasm in one or both ends is frequent, and barred rods are common in certain strains. Granules of protoplasm are gram-positive and take stains deeply; the substrate may be very pale and may even lose a Gram stain. The vegetative forms are usually comparatively chunky in meat medium and also in the muscles of experimental animals. When growing in meat medium, forms are usual in the case of most strains, which on the average are not more than three times as long as thick. Filaments are occasional in animal muscle and in meat medium, they are usual in the serous cavities of animals and reach their greatest size on the surface of the liver of animals. Some strains do not form such filaments until some time after the death of the animal. The orgont forms are extremely polymorphic in shape in animal tissue and are sometimes so in serum mediums, and they are not abundant in meat medium, sporulation occurring in such medium without the formation of large orgonts. "Chunky oval forms" are the common orgonts found in animal tissue. In general the orgont forms are smaller than the corresponding forms in the blackleg group. Spores form early in the development of a culture—in meat medium after, roughly, 18 hours' incubation; they may or may not be present at the time of the death of the animal. Spores are oval, never spherical and never square ended. They vary somewhat in size but usually very little in the ratio of their width to their length. They may be terminal or median in their location in the bacillus; median spores are frequent. Spores are usually symmetrically placed in the rod; two strains (AS and BRH) in my possession form at times spores which are placed distinctly sidewise. The formation of two spores in one rod is exceedingly rare.

3. Cultural Characters: Strictly anaerobic organisms of comparatively energetic growth habit, which show no proteolytic action on meat, serum, or egg mediums, but liquefy gelatin. They produce, in milk, in 1-4 days, gas and a soft clot which, at an early stage, is fragmentable by shaking but later stiffens, and is torn by small bubbles of ascending gas. They split glucose readily producing gas in 18 hours in meat medium and turning it pink. They are not so violently hemolytic as members of the blackleg group. They occasionally auto-agglutinate in liver broth. They are motile in actively growing neutral or alkaline cultures. They reach the zenith of their growth in about 20 hours in most mediums, and after three days no longer proliferate.

Their deep colonies in liver agar are large, quick to develop, and woolly, showing rarely (strains WS and VS), and only early in liver peptone agar, evidences of a lenticular origin; they are subject to variation according to species.

2. Differentiation of the Organisms Involved in Animal Infection: Judged Chiefly by Their Action in the Animal Body

The discussion of the criteria which have been considered as of value in the determination of these groups brings us to the history of the subject.

(*a*) *Differentiation on the Grounds of Putrefactive Action.*—The early history of the vibrion septique and malignant-edema entities is one of the most interesting in bacteriology. Most authors find the descriptions of Pasteur and of Koch too fragmentary to be significant. This I believe to be a mistake. Though it is quite impossible to make any specific determinations on the grounds of the descriptions given by Pasteur and by Koch, it is quite possible to place their organisms approximately. We are, however, forced to pin our sources down to two classic and oft quoted descriptions.

Pasteur's description is significant. He inoculated into a guinea-pig material from a cow that had been dead for three days, presumably of anthrax, and into another, material from a horse that had been dead for one day, presumably of the same disease. We are told only that the disease was suspected and that Pasteur saw anthrax bacilli in the smears. The two guinea-pigs died, showing *désordres epouvantables;* the muscles of the abdomen and of the four legs were extremely "inflamed." Gas was present in the form of pockets, especially in the axillae (*"Ca et là, particulièrement aux aisselles, des poches de gaz"*). The liver and lungs were pale, the spleen normal but at times diffluent and the organisms were present in great numbers in the serosity of the abdomen and about the intestine. They were filamentous in the serous cavities, and regularly became septicemic in the guinea-pig before death. I interpret Pasteur's description to be that of an infection caused by an organism of the vibrion septique group as described on page 389, b. To be emphasized are: extreme inflammation of the muscles, gas production, formation of filaments, infestation of the abdominal serous membranes. septicemia. We are in no position to decide whether either of the two guinea-pigs that were infected from the cow and the horse, and such animals as were infected from them, died of a mixed infection or of a pure one. But surely the infection described by Pasteur was not of the type that shows great contamination by proteolytic organisms, because in such infections gas is not confined in bubbles in the axillae, but forms a large bubble in the destroyed tissue. One is forced to read into such a description as this the things that are not mentioned, as well as those that are. A proteolytic infection in which proteolytic organisms are at all abundant has such an odor of putrefaction that the abductor does not omit to state the presence of such an odor. That other animals which Pasteur inoculated with earth did have a mixed proteolytic infection is of course more than probable. Such infections he later refers to several times: "Un animal va mourir de la putridité septique qui nous occupe, car cette devrait être définie la putréfaction sur le vivant." And: "La septicémie ou putréfaction sur le vivant est-elle une maladie unique? Non; autant de vibrions, autant de septicémies diverses, benignes ou terribles . . ." which shows that Pasteur never pretended that one type of organism was responsible for these infections. This apparently contradicts Perret's statement that Pasteur identified his vibrion septique with the septicemia of Davaine which produced no macroscopic lesions.

It may be concluded, I believe, that Pasteur sometimes worked with mixed inoculum, wherefore he mentioned putrid infections, but that he at other times purified his inoculum by animal passage and that it is from such strains, of which two are included in my collection, that we now have the type of organism that we today recognize as the vibrion septique of Pasteur, which is a strictly nonproteolytic organism. The fact that the *vibrions septiques* of Pasteur, of which we have strains, cause lesions directly comparable with those described by Pasteur on page 793 makes this a type more definite. I do not mean to imply specific determination on the grounds of these old investigations, as I believe specific determinations to be at all times questionable when made from printed descriptions.

The Malignant Edema of Koch: To read the original article in which Koch proposes the name "malignes Oedem" (Zur Aetiologie des Milzbrandes) after spending months in the consideration of the work of his followers who have discussed the subject of "malignant edema" and its causative organisms: to read it in the light of familiar acquaintance with the organisms common in wound infection—is a most illuminating experience. Koch did better work on the subject of anaerobic infection in 1881, with no culture mediums that would grow his organisms, with no Gram stain, with little to guide him, than did any one else till Novy described his B. oedematis maligni II in 1894. The abyss of bacteriologic misunderstanding into which some of Koch's countrymen have today fallen (W. Kolle, Ritz, and Schlossberger, 1918) could all have been avoided by the perusal of Koch's article of 1881, and by the judicious use of laboratory animals. It lies in the nature of things that the malignant edema organisms encountered by Koch were not all the same. But the type described by him on pages 52-56 of "Zur Aetiologie des Milzbrandes" is a type that we know today, and it is not the type to which the vibrion septique of Pasteur belongs. The photomicrographs (table 8, figs. 43, 44, 45) are, however, probably taken from organisms which do belong to the group of the vibrion septique of Pasteur.

At the moment of the writing of his description of the lesions produced by him in experimental animals and termed "malignes Oedem," Koch must have been unfamiliar with the type of lesions described by Pasteur and referred to above. This I judge to be the case because Koch accuses Pasteur of having worked with mixed infections and gives a translation of Pasteur's necropsy findings on the guinea-pigs inoculated from the horse and the cow, allowing himself the liberty of translating "poches de gaz" as "von stinkendem Gas" (noted also by Ghon and Sachs [b, 666]), for which performance I cannot see any justification whatsoever. Koch describes mixed infections following the inoculation of dirt into experimental animals, with their gas and their thin pus-like fluid (Jauche), and he speaks of the peculiar dirty-red coloration of the muscles, which he, quite without justification, interprets to be the color that Pasteur described as due to "inflammation." This type of infection Koch states to be due to a highly complicated mixture of organisms. If one should take this mixture, says he, and inoculate it in any considerable bulk into a new animal, a similar infection will result. But if one should inoculate into a new animal a minute dose from the first animal, one will produce a slower infection, which is caused by a single species of organism. For this type of infection Koch proposed the name "malignes Oedem." It is, I believe, to this description that we must turn for our type of the malignant edema bacillus of Koch, and not to the photomicrographs mentioned above. The infection described is classic. Strange that it should have lain so long forgotten, while the name "malignes Oedem," and the later name B. oedematis maligni Koch, should

have been battered to and fro from laboratory to journal, from journal to textbook, until all semblance of the original pathologic type and of the original organism was lost. Koch's description is as follows: The liquid which fills the subcutaneous connective tissue near the site of inoculation of the experimental animal (sp. ? probably the guinea-pig[6]) is not purulent, but is a pale reddish serum without odor and without gas formation. It contains only one kind of bacilli which in size and shape are almost exactly like anthrax bacilli. (They are a little slenderer and are not grouped in chains like anthrax bacilli [55]). Usually they are nonmotile and only occasionally does one see a movement on the part of an isolated rod. The internal organs of the animal show few changes. The spleen is usually enlarged and darkened, the lung is pale and grayish red. The presence of organisms in the blood varies. The bacilli are short and do not form chains until some time after the death of the animal. They are present in numbers on the serous lining of the abdomen even when no organisms are found in the blood. In mice the organisms regularly become septicemic, in other animals they do not always do so. There are also certain points that must be read into this description. No mention is made of changes in the muscle. If a true pure vibrion septique infection had been seen by Koch at the date of the writing of his description, he would most certainly have mentioned the muscle affection produced by that type of organism, which was described by Pasteur as "inflammation," and he would never have dared attack Pasteur so energetically as he did. Koch does not speak of the consistency of the edema present, he simply calls the disease seen by him "malignes Oedem." Neither does he mention injection of the serous membranes of the abdominal cavity; he insists that the internal organs show few changes; he describes no "désordres épouvantables." The description is so clearly given that one does not hesitate to assign Koch's organism to the same group[7] as B. oedematiens Weinberg and Séguin and B. oedematis maligni II. Novy. The oedematiens strains 139 and Domange of Weinberg and Séguin and strain AB of my own isolation (human gas gangrene) all produce in the guinea-pig lesions exactly like those described by Koch (I have not noted spleen changes); the organisms in my hands sometimes become septicemic and sometimes do not, while organisms of the vibrion septique group always become septicemic, at least in the guinea-pig (this character is, however, not a good one as it is based in my case on cultural determination and in Koch's case on microscopic observation); the organisms enumerated above do not form chains on the abdominal serous membranes of the guinea-pig; they are practically nonmotile, while vibrion septique strains are usually motile; they are large, and resemble somewhat roughly B. anthracis, which organisms of the vibrion septique type cannot well be said to do; most important of all, they do not produce marked muscle lesions or changes in the internal organs as do vibrion septique strains almost without exception; and they produce no gas in the connective tissue of the guinea-pig. I hold most positively that the malignant edema bacillus as described by Koch is nonproteolytic, because Koch states that the liquid near the site of inoculation is "ohne Gestank." This opinion I base on my own necropsy findings. Von Hibler's bacillus X in no way resembles Koch's organism; neither does the organism

[6] As the guinea-pig is mentioned several times on subsequent pages in Koch's paper, and as Koch declares that this animal is extremely sensitive to malignant edema, it is probably safe to conclude that Koch saw the disease in that animal. Whether he saw it in rabbits we do not know, but rabbits are not mentioned in the neighboring text.

[7] By "group" I do not here mean organisms that would necessarily be placed in the same genus by the systematist, but organisms producing the same type of lesions in the animal body.

of Ghon and Sachs resemble it. Koch, so often stated to have identified his organism with the vibrion septique of Pasteur, merely says: "die Oedem-bacillen von ihm (Pasteur) vibrions septiques gennant." As we probably have today no strain called "malignant oedema of Koch," which produced the anatomic picture so well described by Koch, we are not in a position to identify Koch's bacillus specifically with any other organism. We have certainly no right to place it in the vibrion septique group until we have unquestioned strains of the vibrion septique group which produce the lesions described in Koch's classic delineation. But I think we are in a position to place it in the same general group to which B. oedematiens and B. novyi belong. It must, however, be borne in mind that the term "malignes Oedem" was applied to a disease and not to an organism, that Koch and Gaffky used this name for most of their infections that were produced in animals by the inoculation of dirt, and that it is more than probable that among such infections the vibrion septique type was the most usual one. It is unreasonable for us to expect that Koch should differentiate these types. Let us remember when he worked, and under what handicaps. Let us not conclude that Pasteur differentiated his infections with any certainty. Let us remember his confusion of vibrion septique infections with rabies, and remember how few of the followers of Pasteur and of Koch have seen the light in the matter of differentiation of anaerobic infections.

We have here one of those peculiar anachronisms that come about with the lapse of time. Pasteur surely was not clear in his mind as to pure infections and impure infections. He called his "vibrions septiques" putrefactive, but described a nonputrefactive type of infection, and we cannot decide just where his bases of criticism lay. But the strains which have come down to us under the name vibrion septique Pasteur were evidently pure ones and were kept pure in the Pasteur Institute, and we have them today as pure non-proteolytic cultures. Koch, on the other hand, was to my mind perfectly clear as to the difference between putrefactive and nonputrefactive infections; later, he probably isolated his organisms, cultivated them, and gave them to the world. Gaffky also had, for his time, a remarkably clear understanding of putrefactive mixtures and pure infections. To what extent the cultures which were given out were the type described by Koch or the type described by Pasteur, and whether they were of Gaffky's isolation, we cannot tell. The disease described by Gaffky is different from that described by Koch. On page 87 he describes an infection in the guinea-pig entirely similar to that described by Pasteur and he speaks of his organisms as "vibrion septiques." Perhaps it was from one of these guinea-pigs of Gaffky's that the material came from which Koch's photomicrographs were taken. Gaffky also describes another disease of the general type with which we are dealing which was pathogenic for rabbits and mice, and not pathogenic for guinea-pigs. In discussing Koch's organism we are forced to leave Gaffky's organisms out of consideration: it will cause confusion enough to discuss two strains isolated by the same man, let alone those isolated by two men. We must consider that in whatever way we look at these descriptions of Koch's and of Gaffky's, there is an inconsistency between them. These "authentic" cultures of Koch's, if they were pure when given to bacteriologists at large, must quickly have become contaminated, as anaerobe cultures nearly always do in the hands of the aerobist "layman," but they kept their name, "malignes Oedem." And other workers "isolated" other organisms with similar putrefactive contaminations, which produced lesions similar to those produced by the now contaminated cultures of Koch, probably similar infections to those scornfully described by Koch as

mixed. Later von Hibler may have found such cultures, at least he described carefully a proteolytic organism as B. oedematis maligni Koch (von Hibler's X) without the slightest scientific reason for so doing. Weinberg and Séguin (b, 23), and Fraenkel and Zeissler emphasize this point.

Many proteolytic organisms are capable of invading guinea-pig tissue in pure culture when administered in doses of 3 c c or more. This was the basis of Colin's protests against the technic of Pasteur in 1881. I do not wish to be understood as including such dosage in pathogenicity tests. One c c of a 24-hour meat culture is a large dose for a guinea-pig, and I do not employ larger ones.

Ghon and Sachs described an organism from a human case of gas gangrene which they identified culturally with vibrion septique Pasteur. They were unable culturally to compare their organism with the malignant edema bacillus of Koch, as they possessed no culture of Koch's organism. They then patriotically proceeded to call their organism B. oedematis maligni Koch, although the name vibrion septique Pasteur had preference. They did this on the ground of identifications made by German and French workers in the eighties. As Weinberg and Séguin suggest (b, 82), if vibrion septique did not seem appropriate as a name for their organism, surely B. oedematis maligni was no more appropriate, and a new name should have been chosen. The need for such a name was felt, for the organism of Ghon and Sachs was culturally different from the usual conception of B. oedematis maligni, and in pathogenicity it was somewhat different from the usual conception of vibrion septique Pasteur, neither of which organisms had been adequately described, so that later writers (Von Hibler, Meyer, Kitt, Köves, Schlemmer) who have identified organisms with the bacillus of Ghon and Sachs have called it by the name of the describers. These identifications have been numerous because the description given by Ghon and Sachs is detailed and elaborate. Such organisms have usually been defined as nonproteolytic, as forming chains on the liver of animals, and sometimes as nonpathogenic for rabbits. It is, in fact, in recent days, rare to find a record of an infection attributed to the bacillus of malignant edema of Koch, as described by von Hibler, which fact may be interpreted as due to the more careful cultural methods of recent workers.

Von Hibler (a, 88) is emphatic in his statement that the bacillus of Rauschbrand is nonproteolytic. He grouped all organisms found by him which formed woolly colonies, which grew in filaments on the liver of guinea-pigs, and which were nonproteolytic in their action on mediums, under the name "bacillus of Ghon and Sachs." That these strains of von Hibler's were not all of the same species, I am convinced. For example, strain 6 (pl. II, fig. 4) forms on the peritoneal wall of the guinea-pig, 4 hours after death, oval club-shaped forms. Strain 2, which I received in pure culture through the courtesy of Professor W. Frei of Zürich, does not form such club-shaped rods on the peritoneal wall of the guinea-pig, though I have seen 2 other strains of the vibrion septique group that do so in mice. Strain 5 (Kolle u. Wassermann IV. pl. XIX.1) does not form such clubs on the liver of the guinea-pig 6 hours after death. I am emphatically of the opinion that this type of character is specific. Von Hibler grouped strains that formed woolly colonies and that grew in filaments on the liver of animals but which were proteolytic in their action on mediums, under the name B. oedematis maligni Koch. Weinberg and Séguin (b, 24) seriously criticise von Hibler for this classification, saying that his identifications are incomplete, adding that von Hibler never isolated a strain from man that corresponded to this conception, all his human cases of "oedème malign" being attributable to the bacillus of Ghon and Sachs.

Von Hibler isolated his strains of B. oedematis maligni from a mule, from garden earth that was inoculated into an animal, and from a cow that died of puerperal septicemia. Of his cases that were attributable to the bacillus of Ghon and Sachs, 5 were from man and 2 were from cattle. I do not understand why Weinberg and Séguin insist that an organism of this type should come from a human case in order to be genuine. I have, however, in a series of some 200 samples of pathologic material from animals and man, never encountered any strain which corresponds to the bacillus of malignant edema as described by von Hibler. That the strains studied by von Hibler were occasionally impure is witnessed in mute testimonial by two photomicrographs (a, pl. XI, figs. 2 and 16) which unquestionably portray mixed cultures.

Kitt (e, 287) classes as one species most of the anaerobic organisms that infect wounds, and he gives to this conception the name used for the organism of Koch, in its Latinized form, B. oedematis maligni. Of the agar cultures of this organism he states: "Das Gas ist geruchslos. (Von einigen Autoren wird angegeben, das stinkende Gase sich entwickelen; Mischkultur? Verwechslung mit anderen Kadaverbacillen?") He mentions here no proteolytic invaders. But in discussing Rauschbrand (e, 301) he declares that milk may be used to differentiate Rauschbrand from malignant edema, as the latter liquefies it and produces a foul odor (Smith, E. von Hibler). He describes a mode of obtaining B. oedematis maligni by inoculation of earth into mice or guinea-pigs, and holds that if such an infection is a mixed one, another inoculation will give a pure culture of the malignant edema organism. My experience with such infections has been very different from Kitt's. Earth contains many species of anaerobes that will infect mice and guinea-pigs, and mixed infections may occasionally persist as such after several passages, though if a true organism of the vibrion septique group is present, it usually outruns all others in the first or second passage. Von Hibler describes several species of invasive anaerobes that were isolated in this manner. Kitt mentions the fact that spontaneous malignant edema infections are often mixed, but he does not mention any anaerobes as being contaminating organisms.

Hutyra and Marek (33) say of B. oedematis maligni: "Milk is changed in a few days into a watery fluid with gas formation and the collection of a layer of fat on the surface (Smith)"; and they say (41) concerning the blackleg bacillus: "Milk is curdled only imperfectly."

Lehmann and Neumann (500) state that a putrid odor is indicative of malignant edema and absence of such odor is to be taken as indicative of Rauschbrand.

Bongert (198) declares the bacillus of malignant edema produces "höchst übelriechende Gase verschiedener Art."

Grassberger and Schattenfroh (a and b) declare the Rauschbrand bacillus is an exquisite butyric acid bacillus (nonproteolytic), and the malignant-edema bacillus (vibrion septique) is a proteolytic organism. (See, however, reprint.)

Von Werdt (a and b) recognizes the existence of a "true bacillus of malignant edema of Koch" which is proteolytic and to the discussion of which he devotes a chapter: "Malignes Oedem." He also recognizes the existence of a group of organisms which he calls the organisms causative of "Gasbrand" and to which he devotes another chapter: "Gasbrand und seine Erreger." These organisms comprise: Welch-Fraenkel's bacillus, Ghon-Sach's bacillus I., Novy's bacillus, von Hibler's VI, VII, and XI, Ghon-Sach's bacillus II, Klein's B. enteritidis sporogenes, Klein's B. cadaveris sporogenes, Markoff's pseudo-rauschbrand organisms, the bacillus of malignant emphysema of Stolz, the

bradsot bacillus, and the whale septicemia bacillus. These organisms are extremely varied in their affinities and in their pathologic action; some are proteolytic and some are nonproteolytic. Von Werdt's work is in the nature of a compilation only, and in no way tends to clarify the confusion existing in the anaerobic field. It reflects exactly the history and literature of the subject, and may be consulted for valuable details and for its excellent bibliography. It it not critical.

Foth (b, 252) conceives Rauschbrand to be nonproteolytic and the chain-forming organisms to be proteolytic. His chain-forming strains vary in proteolytic action, but he has never found a chain-forming strain which in its behavior approaches the nonproteolytic character of the bacillus of Ghon and Sachs. Foth uses the term "verbandbildende Anaeroben" to include various species.

Wulff, who worked in Foth's laboratory, has a conception similar to that of Foth and calls all proteolytic infections "malignes Oedem."

Markoff divides his strains, as does von Hibler, into blackleg, nonproteolytic chain formers, and proteolytic chain formers (malignes Oedem).

Weinberg and Séguin (93) declare vibrion septique to be nonproteolytic. Robertson (a) emphasizes the same point. Neither reports any such organisms as the malignant edema of Koch-von Hibler.

Eugen Fraenkel and Zeissler, who carefully isolate their strains, have never found such an organism as the malignant edema bacillus of Koch as described by von Hibler.

In the light of my own experience with anaerobes, I must state that until I find in my hands an indubitably pure culture of a highly proteolytic organism, which alone forms filaments on the liver of a guinea-pig and which has more than a history of pathogenicity, I must continue to doubt the identity, nay, the very existence, of B. oedematis maligni Koch, as described by von Hibler. No one would be more interested or more delighted to find and study such a strain than I. If the organism does exist, I think I am safe in stating that many identifications of it have been faulty, and were based on the investigation of impure cultures. All through the anaerobic literature we find references to pathogenic proteolytic organisms and some of these no doubt exist as entities, capable of penetration in pure culture. Such organisms are, however, exceedingly rare. I have found only two such strains in a series of a hundred tissue invading anaerobes, and these two are only slightly proteolytic. I wish at this point to express my thanks to Miss Muriel Robertson, under whom my anaerobic studies began, for the principles regarding anaerobic investigation which she taught me. It was from her that I learned to discredit the purity of a proteolytic pathogenic culture till I had investigated both the purity and the pathogenicity of such a culture with all the resources at my command. Practically all strains that have come into this laboratory with a history of pathogenesis and that have shown a proteolytic action on meat proved either to be no longer pathogenic for guinea-pigs or to contain a primary invader in the form of a nonproteolytic organism.

I have found no proteolytic invaders in cattle, sheep, or hog material.

I wish also to state at this point that in my hands the characters of anaerobes change no more frequently than do those of other organisms. When a culture radically changes its habit it will be found to contain more than one species of organism.

We may sum up the situation by stating that because of the universal occurrence of organisms of sporogenes affinities and because of their frequent contamination of pathogenic cultures, and because of their occasional invasion of living tissue in company with nonproteolytic invaders, "malignant edema" of animals and man has frequently been described as due to a proteolytic organism.

Friedberger and Fröhner (178) and authors of other textbooks emphasize the universal occurrence of the malignant edema organism, apparently confusing it with the ubiquitous proteolytic forms. They say: (189) "Mit dem Fortschritte der Faulniss verlieren die Oedembacillen ihre Wirksamkeit. Nach etwas 2 Monaten hört sie ganz auf. Trocknet man die Bacillen jedoch bei 15—38 c., ehe Faulniss aufgetreten ist, so bewahren sie ihre virulenz dauernd." Of the fact that proteolytic organisms so overgrow pathogens as to render them no longer pathogenic or to eliminate them entirely, I could give a hundred examples from my own findings. Weinberg has shown that the filtrate of B. sporogenes neutralizes the toxin of B. oedematiens in vitro and neutralizes to some extent even the toxin of vibrion septique, but that it does not neutralize the toxin of B. perfringens (B. welchii). Certain workers (Donaldson and Joyce, Mayo-Robson) have even used cultures of organisms of the sporogenes type in the treatment of wounds in man.

Let us for practical purposes group the vibrion septique of Pasteur, the bacillus of Ghon and Sachs, and whatever organisms resemble them, under the name of "the vibrion septique group." A definition of this group is to be found on page 389, b. May I be forgiven if, solely in reviewing the literature, I sometimes use, in conformity with an author, the term "bacillus of malignant oedema" as applying to a proteolytic strain?

Although the traditional idea has been that B. chauvoei is nonproteolytic and may by this sign be distinguished from B. oedematis maligni, some authors have attributed a proteolytic action to it. Sperry and Rettger refer to the well-known putrefactive organisms B. anthracis symptomatici and B. oedematis maligni. McCrudden states that B. chauvoei putrefies fibrin. Many older authors do not seem to be very clear on this point, or they make no mention of it. It is a misconception exactly like that concerning the organisms of the vibrion septique group as dealt with by many workers.

(*b*) *Gas Production in the Animal Body.*—This has sometimes been referred to as a distinguishing character.

Gas production depends on the amount of bacterial proliferation in the tissues, and is a relative matter for each separate wound infection, depending on the site of the wound, its extent and its isolation, and on the toxicity of the specific organism for the specific or individual host and also on the size of the host. The more toxic a strain is for the host, the less bacterial proliferation will there be before the death of the animal, and the less gas will there be formed. Furthermore, a bacterial process may exist longer in a large animal before producing death than it can in a small one. All strains produce gas in meat medium. I find that, given a uniform technic, strains of the vibrion septique group produce distinctly more gas in the muscles and connective tissue of the guinea-pig than is produced by members of the blackleg group. An exception to this statement is the behavior of the vibrion septique group strain which I isolated from whale muscle (p. 443), which is so toxic that it kills before it multiplies to any great extent, and produces little or no gas in the guinea-pig

and rabbit. I have observed also that far more gas is to be found in the intestines of guinea-pigs dying of vibrion septique infection than of those dying of blackleg infection.

(c) *Formation of Chains or Filaments on the Liver of Animals.*—This has often been used as a criterion of distinction between the two groups. The malignant edema or vibrion septique strains form filaments and the blackleg strains do not. I find, however, that the mode of filament production and sporulation after filament production varies with the species of vibrion septique organism, with the dose culture, with the time elapsing before necropsy, and with the species of animal on whose liver the filaments are produced. I have never observed the formation of chains on the liver of animals dying of a blackleg infection.

(d) *Appearance of Infiltration and Necrosis.*—The appearance of the infiltration and necrosis (gaseous hemorrhage or serohemorrhagic) in the muscles of the host is also used as a differential character. I find the character and degree of the hemorrhagic exudate and the color of the muscle a good lesion to use when standardizing my technic on the guinea-pig. Blackleg almost invariably produces an intense black coloration in spots or in a circumscribed area in the muscles and connective tissue, while vibrion septique strains do not. The latter organisms are, however, distinctly hemolytic in their action, and it would sometimes be very easy for the inexperienced to mistake the coloration produced by them for that called forth by blackleg. It must also be borne in mind that the production of hemorrhagic exudate is a relative matter, dependent on the specific action of the hemotoxin on the corpuscles of the host and on the amount of bacterial proliferation, which in turn is dependent on the location and nature of the wound or focus, on the degree of the toxicity of the specific organism for the specific host, and on the size and age of the host.

(e) *Alteration of the Serous Membranes of the Abdominal Cavity.*—This should always be considered in distinguishing between organisms of the vibrion septique group and those of the blackleg group. The tendency to cause injection of the intestines and of the peritoneum is very great in the case of the former and very slight in the case of the latter. This is in the guinea-pig an almost determinative character.

(f) *Presence of the Pathogenic Organisms in the Bile.*—This has been used by a few authors as a means of differentiation. Foth (b, 212) found this a criterion of no value. Wulff (706) found the method wholly unreliable.

(g) *Depilatory Effect of the Inflammatory Process.*—This is a relative character of a little value. It is dependent on the degree of bacterial multiplication in the skin, and is most marked in proteolytic infections, and least marked or quite absent in highly toxic vibrion septique infections. Blackleg infections often show it, and combined with it is frequently a serous exudate. The animals that die most slowly have the greatest bacterial multiplication and their hair is most easily removable.

(h) *Delimitation of the Zone of Infection.*—This is not an absolute character. In general, the zone macroscopically affected in blackleg infections is more sharply limited in outline and extent than the zone affected in vibrion septique infections.

(i) *Speed With Which Death Ensues.*—This varies of course with the dose inoculated. Given the same technic, almost all vibrion septique strains kill guinea-pigs before the usual time taken for death to intervene in the case of blackleg infections. Guinea-pigs infected with a dose of 0.5 c c of a 24-hour

culture of a vibrion septique strain die in about 16 hours, those infected with the same amount of blackleg culture die in approximately 20 hours; but there is great variation in these figures. It must also be borne in mind that a blackleg culture does not contain as many organisms as does a vibrion septique culture.

Many authors mention the *characteristic odor* produced in the animal body by blackleg infections. I have not detected any difference in the odor of guinea-pigs dead of the two diseases, but perhaps my perception is at fault.

(j) Minor Characters Common to Both Types.—Both types of organisms tend to cause profound liver disturbances in the guinea-pigs, which are shown by bile staining of the intestines and frequently by an emptying of the gall-bladder. Both types may cause the formation of hemorrhagic erosions on the stomach lining of the guinea-pig, and both may cause enlargement and injection of the suprarenals. Foth (a, 204) and Wulff (45) state that both types produce ochre yellow foci in the liver of cattle. Meyer (c, 684) found infarcts in the liver of cattle dying of atypical B. chauvoei infection, and (b) isolated vibrion-septique-like organisms from liver foci of cattle dying of an obscure disease in the mountain valleys of California. Both types may invade the pleural cavity and even the lungs; both become septicemic in the guinea-pig, with practically no exceptions, and may be isolated from the heart blood. Both types produce a serogelatinous edema of the connective tissue spaces, and a liquid infiltration of the muscles; they soften somewhat the muscles in which the organisms multiply extensively.

(*k*) *Characters Possessed by Neither Type.*—Neither type of organism produces, when inoculated in pure culture, a greenish discoloration of the skin, a foul or putrid odor, tissue disintegration, or a brownish, a grayish, or a yellowish discoloration of the muscles. Neither type produces a gelatinous edema of the connective tissue spaces that retains its gelatinous consistency long after section, nor do they produce an edematous infiltration of the muscles. Though the organisms multiply actively on liver, the phenomenon of "foamy liver" is probably usually a postmortem one.

(l) Pathogenicity for Certain Species.—Pathogenicity for certain laboratory animals, notably for the rabbit, is frequently cited in the older textbooks as a final criterion when one is in doubt as to the nature of an organism under consideration. Malignant edema was thought to be pathogenic for the guinea-pig only in spite of the fact that the bacillus of Ghon and Sachs, generally known to be of the same group as vibrion septique, is not pathogenic for rabbits. Meyer's hog bacillus is relatively nonpathogenic for rabbits, and Meyer (a) emphasizes the fact that "any conclusions drawn from such (pathogenicity) tests would be entirely erroneous without the necessary microscopic examination and anatomic studies." When one considers how greatly the technic of various workers differs in making pathogenicity tests, and when one bears in mind the fact that different individuals of a species vary in their susceptibility to infection, one is ready to agree heartily with Meyer's statement as to the secondary importance of such tests.

(m) Epidemiologic Considerations.—Most deceptive, and, may I venture to say, crudest, of all differentiations between the two groups of organisms are those based on epidemiologic grounds. Diagnosis of the nature of a disease according to whether it is endemic in a locality or whether it is rare in that locality is an unscientific proceeding. Specific diagnosis of an infection, which is based on the presence of a wound as a portal of entry, on the history of a recent parturition, or on the age of the animal attacked, is likewise unjustifiable.

3. CULTURAL AND MORPHOLOGIC DIFFERENCES BETWEEN THE ORGANISMS OF THE TWO PRINCIPAL GROUPS

(a) Motility.—The motility of blackleg and vibrion-septique-like organisms is given differently in different works. Both types (Arloing, Cornevin, Thomas; Pasteur) were originally described as motile. I find that in an open drop on an unwarmed slide the vegetative rods of the blackleg organisms in a 24-hour meat culture may be sluggishly or actively motile, moving in a dancing head-over-heels fashion, while under the same conditions, organisms of the vibrion septique group are noticeably but sluggishly motile, moving occasionally in the manner of a boat or even sidewise, but not tumbling or dancing.

(b) The Gram Stain.—The Gram stain is used to differentiate the two groups. Lehmann and Neumann (500) give malignant edema as gram-negative and blackleg as gram-positive. Bongert says that malignant edema is gram-negative. Friedberger and Fröhner give both as gram-positive. Von Hibler (a, 13) states that all anaerobes studied by him are gram-positive but that the Ghon-Sachs bacillus and the malignant edema bacillus are the most easily discolored by the alcohol. Foth (a and b) declares both types to be gram-positive. My experience runs counter to most of these statements. I prefer a carbol-methyl-violet (6BN) Gram stain [8] with slight decolorization or a thin carbol-gentian-violet Gram stain, which requires longer decolorization. With either of these stains vibrion-septique strains are usually gram-positive and blackleg-group strains are gram-negative.

(c) The Resistance of Spores to Heat.—This has been used by von Hibler and by some of his predecessors (a, 217) as an anaerobic character. I doubt if spore-resistance could be used to distinguish blackleg strains from vibrion septique strains—and we have many characters to use for differentiation that are more definite.

(d) Morphology.—Opinions vary greatly as to the value of morphologic characters for the differentiation of blackleg from the vibrion septique group. This confusion I attribute solely to the reigning confusion as to the identity of the strains studied. One reason for this state of affairs is that there still are diagnosticians who call an organism blackleg because it comes from a cow. But another important reason is the existence of a large number of contaminated strains in circulation among laboratories. The usual contaminators of sporogenes and bifermentans type, or of tertius or putrificus morphology, naturally upset all morphologic conceptions. Moreover the blackleg organisms in their growth in muscle resemble the vibrion-septique organisms growing in muscle more closely than either type resembles most other anaerobes. This does not mean that they are alike or indistinguishable.

I may sum up the value of morphologic determination according to my own experience as follows: If the worker is unfamiliar with anaerobic technic and with the anaerobic group as a whole, his opinion (beyond his observation of filaments on the liver of an animal) is worthless. And if the worker has not an eye capable of distinguishing with some certainty a contamination in a smear of an anaerobic culture in vitro or in an animal, his opinion is worthless. But for the trained anaerobist, employing a technic with which he is familiar, the organisms of these two groups are, in morphology and in staining reaction, highly characteristic, and they are not to be confused. I could under-

[8] Two and five tenths c c saturated alcoholic solution of stain in 100 c c of 5% pure phenol; dilute 1: 10, filter, apply for 2 minutes, wash, apply Lugol's iodin 1 minute, wash with 96% alcohol, once across the surface of the slide for methyl violet (8 drops), twice across the surface (12 drops) for gentian violet. Counterstain with dilute 1: 10 carbol fuchsin.

take to teach any observing bacteriologist the difference between the two types in a few hours. I would suggest that those interested in this subject study carefully pure cultures of organisms of the two groups in the guinea-pig and in meat medium with a weak or carefully decolorized Gram stain (see p. 401, b) Forms are occasionally seen which may be referred to either group; morphologic criteria could not free a suspected mixed blackleg-vibrion-septique culture of suspicion, but the general picture of the cultures and of the smears of animal exudates containing these two types is entirely different, and any one can recognize this difference on his own mediums and with his own stain as soon as he is acquainted with both types.

(*e*) *General Cultural Characteristics* are not of great value in distinguishing the two groups. In general the organisms of the blackleg group are not so active in their growth on artificial mediums as are those of the vibrion-septique group; they occasionally fail to grow on a medium on which vibrion-septique organisms will readily grow. For example, I have repeatedly failed with massive inoculation to induce them to grow on brom-cresol milk. But in gross biologic determinations the two groups are so closely related that they are thrown together rather than separated. However, gross determinations separate these groups from other groups, especially from the Welch group and from the oedematiens group. Finer biologic determinations may be of great value, not only in differentiating the blackleg organisms from the vibrion-septique organisms, but also in differentiating distinct species within these groups. So far as my experience in the matter goes, the more detailed and more or less quantitative cultural tests, combined with morphologic characters, are of more value in differentiating these organisms than are immunologic tests.

(*f*) *Colony Formation.*—Colony formation has not been used by many recent authors as a means of differentiation between the two groups. I have, however, found colony formation in agar under carefully controlled conditions to be of immense value in distinguishing, not only between the blackleg and vibrion-septique groups, but also between the individual species in these groups.

(*g*) *Carbohydrate Fermentations.*—Carbohydrate fermentations have occasionally been resorted to as a means of differentiating anaerobes.

Achalme analyzed the behavior of vibrion septique and the bacillus of symptomatic anthrax on sugars. As his cultures were proteolytic I shall not quote his results.

Smith used fermentation tests on three sugars to distinguish his anaerobic strains. The nonproteolytic strains called "Rauschbrand" behaved consistently; the proteolytic strains called "edema bacilli" behaved differently from the nonproteolytic ones and consistently with each other.

Bahr made a more extensive study. He divided his strains into 5 groups, the first group containing 9 strains of quite different origin: sheep, hogs, cattle and a horse; they were from different countries. The other groups contained one strain each. Bahr isolated his strains by animal passage and a colony method.

Grosso studied carbohydrate fermentations. His isolations were carefully made.

Meyer (a) found his hog strain, a vibrion-septique strain, and a blackleg strain to behave alike.

Todd worked out an elaborate scheme for sugar determinations on this group, but some of his cultures were proteolytic and the work cannot be quoted.

TABLE 1

Table of Carbohydrate Fermentations as Quoted from Various Workers

	Glucose	Levulose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Mannose	Xylose	Rhamnose	Arabinose	Amylum sol.	Inulin	Cellubiose	Glycogen	Salicin	Amygdalin	Trehalose	Mannite	Dulcite	Quersite	Sorbite	Glycerin	Adonite
Smith's nonproteolytic	+	..	..	—	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Smith's proteolytic	+	..	..	+	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bahr's group I 3 Bradsot 2 Oedematis maligni 1 Pseudo-Rauschbrand horse 3 Rauschbrand cattle	+	..	..	—	+	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—	..	—	+	—
Bahr's group II Rauschbrand, Kitt	—	..	..	—	+	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—	..	—	+	—
Bahr's group III Bradsot, Scotland	+	..	..	+	+	+	..	..	—	—	—	..	..	..	..	.	..	..	—	—	..	—	—	—
Bahr's group IV Rauschbrand, Ostertag	+	..	..	+	+	+	..	..	+	—	+	..	..	..	..	.	..	..	+	—	..	+	+	—
Bahr's group V Malignant edema, Halle	+	.	..	+	+	+	..	..	+	+	+	.	..	..	..	.	..	..	+	+	..	+	+	—
Grosso's group I 2 Malignant edema 1 Bradsot	+	..	..	—	..	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—	—	..	—	—
Grosso's group II 2 Pseudo-Rauschbrand	+	..	..	+	..	+	..	..	—	—	-	..	..	.	.	..	..	.	—	—	—	..	—	—
Grosso's group III Rauschbrand	+	..	..	+	..	+	..	..	—	—	—	.	..	..	..	..	..	..	—	—	..	..	+	—
Meyer's hog bacillus Vibrion septique Rauschbrand 2, Munich	+	..	..	—	+	+	..	..	—	—	—	..	..	.	..	.	..	..	..	—	..	—	—	..
Robertson, several Vibrion septiques	+	+	+	—	+	+	..	..	..	..	.	..	—	..	..	+	..	..	—	—	..	..	—*	..
Robertson's B. chauvoel	+	+	+	+	+	+	..	..	..	..	..	..	..	..	.	..	..	..	.	..	..	..	—*	..
Christiansen's whale septicemia bacillus	+	+	+	—	+	+	—	+	—	—	—	—	—	+	—	+	—	+	—	—	—	—	—	—

* Med. Res. Committee, No. 39.

Robertson found vibrion-septique strains behaved alike, and a B. chauvoei strain behaved differently from them.

Christiansen made extensive determinations on his whale septicemia bacillus.

One observes a general tendency on the part of anaerobes of these groups to split the hexoses and the disaccharids, and to leave the higher carbohydrates untouched. Smith's nonproteolytic group, Bahr's group 1 (9 strains), Grosso's group 1, Meyer's 3 organisms, Robertson's vibrion septiques, and the whale septicemia bacillus of Christiansen are wholly consistent, and may be taken as characteristic of the vibrion-septique group. They may be signalized as not splitting saccharose. The remaining strains referred to B. chauvoei are not so consistent in their behavior; Grosso's and Robertson's strains split saccharose. There are not enough data from which to draw further conclusions. I hope to perform extensive fermentation tests with my strains in the near future. It would appear that a satisfactory basic medium for carbohydrate determinations for use with anaerobes is yet to be found.

4. DIFFERENTIATION BETWEEN THE TWO PRINCIPAL GROUPS BY MEANS OF IMMUNE REACTIONS

(*a*) *Immunization in General.*—The anaerobes with which we are dealing are antigenic organisms par excellence. Immunization experiments have been carried out by many workers, in almost as many ways. Results are apparently contradictory, but on careful scrutiny they are found to be consistent. Immunization of guinea-pigs has been accomplished with cultures, washed or heated organisms, and organisms chemically attenuated; with toxic and even nontoxic filtrates of cultures and of infected tissue; also with serum from immunized animals.

(*b*) *Active Immunization with Cultures.*—We shall deal here chiefly with immunization work which was performed with the purpose of distinguishing our two important types. I find the following notes as to the active immunization of laboratory animals by means of cultures:

Roux in 1888 used culture which had been heated at 115 degrees for immunization purposes.

Kitasato in 1889 found that a two-weeks-old culture of B. chauvoei in guinea-pig broth could be used for the immunization of guinea-pigs, as could a heated culture.

Le Clainche and Vallée found that guinea-pigs immunized by attenuated blackleg virus were not immune to vibrion-septique infection.

Markoff used cultures in 0.5% sodium formate broth with 2-5% glucose and found immunization of guinea-pigs with blackleg and chain-forming organisms possible with such cultures.

Wulff (612) used dry powdered infected muscle for immunization.

The authors referred to above all agree that blackleg and the chain-forming organisms may be distinguished by this means.

(*c*) *Types of Toxin.*—The best known anaerobic toxins are those of the proteolytic organisms B. tetani and B. botulinus. These toxins are apparently produced slowly in a medium, and increase in power as incubation continues. They are pathogenic in extremely minute doses. It is of interest that the more proteolytic a pathogen is, the more powerful is its toxin, the less

energetic its invasive properties. The toxin of B. oedematiens, which has mild proteolytic properties (Med. Res. Comm. No. 39, 63), is less powerful than that of B. tetani and that of B. botulinus which is the most highly proteolytic of the three, but it is far more powerful than that of the vibrion-septique and Welch-group types, which are nonproteolytic organisms (Med. Res. Comm. 39, 116).

(*d*) *The Aggressin Test.*—The determination of toxin and antitoxin relationships is of immense practical value. Tulloch has shown in his tetanus work that aggressins are antigens which must also be considered in anaerobic infection. Probably cultural methods can be made to supersede the agglutination method entirely, because immunologically it has no practical significance. But the toxins and the aggressins have great practical significance: What does it matter to the therapeutist if two strains can be shown to belong to two different species by colony formation or other delicate methods, so long as they produce the same toxin and aggressin? A practical method of arriving at the combined toxin-aggressin factors would be by means of the immunization of guinea-pigs with young whole culture, after a preliminary inoculation of killed organisms. More delicate methods of demonstrating aggressins are two: (1) The delicate phagocytic methods in use in Sir Almroth Wright's laboratory may be used to show the action of bacterial products and their antibodies on leukocytes; or (2) accurate animal experiments may be turned to, filtrates of definite age from mediums of standard composition being used.

Immunization by means of filtrates was undertaken in 1888 by Roux, who found that filtrates of cultures of vibrion septique and B. chauvoei were excellent for immunizing guinea-pigs. He found filtered serous exudate of guinea-pigs dead of vibrion-septique infection to be more highly toxic for guinea-pigs than that of guinea-pigs dead of blackleg infection, and he found such serosity to be a good immunizing agent. By such a means he immunized guinea-pigs against both diseases and found that those immunized against vibrion septique were not immune to symptomatic anthrax, but that those immune to symptomatic anthrax were immune to vibrion septique. Kitasato repeated Roux's experiment and found that Rauschbrand did not protect against malignant edema.

Schöbl (1910) found that centrifuged edema fluid of an animal dead of blackleg infection immunizes guinea-pigs against blackleg inoculation. This method has received wide practical application in the immunization of cattle.

Nitta (1918) found that blackleg culture filtrate furnished adequate protection to cattle.

(*e*) *Passive Immunization.*—More extensive work has been done in the way of differentiation by means of passive immunization.

Duenschmann, in 1894, attempted to demonstrate immunologically the relationship between vibrion septique and B. chauvoei. His immunization strain, called B. chauvoei, which he received from Arloing, who used it for vaccine, was, however, a member of the vibrion-septique group. This was pointed out by Le Clainche and Vallée (595). Duenschmann considered it to be a true B. chauvoei because it did not kill rabbits. It killed guinea-pigs rapidly "with all the well-known signs of symptomatic anthrax" (details not given); it sporulated heavily in the animal body, many spores being median; the bacilli were short, but formed long motile filaments on the peritoneal wall. Duenschmann found that the serum of rabbits immunized with blood cultures of this strain immunized guinea-pigs against a stock vibrion-septique strain of the Pasteur Institute.

In 1900 Le Clainche and Vallée studied immunologically the relationships between B. chauvoei and vibrion septique. They found that a B. chauvoei serum which protected guinea-pigs against B. chauvoei inoculation, would not protect against several strains of vibrion septique, all of which were protected against by one vibrion-septique serum.

Foth (a, 254) used serum of rabbits immunized by injection of cultures of Rauschbrand bacilli. This was given subcutaneously to guinea-pigs in one thigh, culture was given on the other; and animals that were protected, while controls died with lesions of Rauschbrand, were considered to prove the existence of Rauschbrand.

Kitt (a) says that serum immunization may be used to differentiate Rauschbrand from the infections that simulate it.

Markoff preferred serum immunization of guinea-pigs to culture immunization, and found that it divided his organisms into fundamental groups.

Wulff (612) found serum immunization a diagnostic aid of great value.

Differentiation of blackleg from malignant edema by means of toxin-antitoxin determination has been undertaken by Grassberger and Schattenfroh (b and c). They hold this procedure to be the only fundamental means by which to distinguish the two types from one another, which is entirely comprehensible when one considers their technic. They use an inoculum of dried juice from an animal dead of blackleg, which is confessedly impure, which Grassberger and Schattenfroh hold to be unimportant. They then place a piece of this material in a tube containing a chunk of "sterile" beef muscle, taken aseptically from a larger piece (Foth [b, 241] and Conradi claim that such muscle is seldom sterile), pour over it 2-3% glucose broth, incubate in a Buchner tube and in 24 hours find that microscopic examination shows sporulating and nonsporulating forms of "half denatured" Rauschbrand bacilli! They find that this broth is highly pathogenic (unfiltered?) for rabbits, and that a highly potent antitoxin will protect the rabbits. Likewise, malignant edema forms a toxin which is specific.

I have every confidence that the toxins of blackleg and of vibrion-septique strains will be found to be specific—what we need to know is whether all toxins produced by the members of one group are alike (see p. 427). I wish, however, most emphatically to protest against such gross methods as those of Grassberger and Schattenfroh. Immunologic work cannot be regarded as serious when it is performed with impure cultures. It is especially necessary to have pure cultures with which to produce immune serums, but Grassberger and Schattenfroh do not mention any precautions taken in this matter. Especially is this fact to be considered as entirely disqualifying the work of Grassberger and Schattenfroh when one considers the fact that the usual "denatured form" of Rauschbrand described by Grassberger and Schattenfroh is the ubiquitous B. Welchii (B. phlegmones emphysematoseae [von Hibler a, 5]), and that that organism produces a highly potent toxin in the type of medium described by Grassberger and Schattenfroh.

Meyer (a) found that serum immunization sharply distinguishes the groups.

Nicolle, Cesari, and Raphael demonstrated by means of antiserum the identity of the toxins of various tissue-invading strains of animal origin. They found also that their antiserums protected against cultures of the various strains. These strains comprised one strain from the blood of a cow, which was putrified, one from the cadaver of a rabbit "altéré," and two from the cadavers of guinea-pigs "altérés"—these strains Nicolle, Cesari, and Raphael called vibrions septiques. The authors studied also several strains that they call B. chauvoei, one of which came from a hog and the others from cattle

I can find, however, no evidence in their protocols that they worked with any typical strains of B. chauvoei. They repeatedly insist that their strains all behave alike except for some differences in pathogenicity, the organisms all kill rabbits if the dose given is as much as 1 c c, and all apparently produce "bulles gazeuse" in the animal body, and congestion of the intestines; the black hemorrhagic exudate so typical of blackleg is not mentioned. It is impossible to believe that differences so striking as those noticed in the pathology of the two diseases in the guinea-pig body would have been passed over by these workers had they been seen. The identification of the strains of hog and cattle origin was probably referred to the donors of the cultures. Three of these strains were from Dr. Vallée. I find that one of Vallée's classic B. chauvoei strains belongs to the vibrion-septique group. I am at a loss to account for this as it is apparent that Le Clainche and Valée were entirely aware of some of the distinctions between the two types. Le Clainche and Vallée state, however, that it is difficult to keep a strain of B. chauvoei from becoming displaced by vibrion septique through animal passage. I have never known this to happen with my technic, though it is quite conceivable that it might happen if peritoneal or subcutaneous serous exudate were used for purposes of inoculation.

A recent piece of work is that of Klose (c). Klose studied the protection afforded to mice and guinea-pigs against the toxins of anaerobes by antitoxic serums. The purity of Klose's strains would bear investigation. He divided them into two groups, the putrificus (proteolytic) group, and the Rauschbrand group; in the latter are included all strains which formed a toxin that was neutralized by a "Rauschbrand" serum of the "Höchster Farbwerke," and also the organisms which culturally resemble the Ghon-Sachs bacillus. His results show that the Höchst "Rauschbrand" serum must have been made from a vibrion-septique strain. Klose investigated 4 strains and 5 serums. Two strains were distinctly proteolytic (K16b and KI); one (Ficker I) partially liquefied serum but was relegated to the nonproteolytic group, and one was nonproteolytic (Berlin). The serums "Gas-Oedem Höchst Op. 3," "Rauschbrand Höchst," and "Goldfuchs" from v. Wassermann, all protected against the nonproteolytic strain, the slightly proteolytic strain, and one of the highly proteolytic strains. They did not protect against the highly proteolytic strain KI, and the antitoxin of strain KI did not protect against the other strains. The pathogenesis strain KI may be assigned to the oedematiens group, as it resembles B. novyi (a). Landau showed that the Höchst serum did not agglutinate two of Kitt's Rauschbrand strains. I doubt if any true Rauschbrand entered this complex. The matter of proteolysis I lay to contaminations. Toxin determinations will probably prove to be excellent for the practical differentiation of these organisms.

Klose (a) studied elaborately the toxin of a highly pathogenic anaerobe that resembles the B. oedematis maligni II of Novy. He insists (b, 293) that immunologic methods, especially toxin-antitoxin determinations, are fundamental criteria for specific determination of anaerobes. Zeissler (a, 489) finds Klose's strains of pathogenic anaerobes contaminated with proteolytic organisms and attacks Klose for placing "putrificus" strains in the Rauschbrand group on the basis of toxin-antitoxin determinations. Zeissler calls the value of toxin-antitoxin determination seriously into question. Of course, if one is to use impure cultures in such determinations one should not make the mistake made by Klose of confounding the cultural characters of the contaminators with those of the toxin-forming organisms.

Various workers have produced toxins from the organisms of the groups under discussion, though Haslam and Lumb showed that blackleg culture filtrates made by four different laboratories possessed immunizing properties but no toxic power for the guinea-pig. These authors were careful as to the identity and purity of their cultures. I should not consider that their work shows that blackleg organisms do not produce toxin, since the cultures used were not young ones.

(*f*) *The Agglutination Test.*—The agglutination reaction has naturally frequently been utilized as a means of differentiating anaerobes.

Le Clainche and Vallée sharply differentiated B. chauvoei from vibrion septique by means of the agglutination reaction.

Hillbrand differentiated bradsot from malignant edema by agglutination.

Grosso discusses the question. He found that all bradsot strains were agglutinated by the same monovalent serum, which also agglutinated all his malignant-edema strains. He therefore pronounced the malignant-edema organisms to be the cause of bradsot. His agglutinations were consistent, as follows:

Bradsot serum agglutinated malignant-edema cultures.
Malignant-edema serum agglutinated bradsot cultures.
Rauschbrand serum did not agglutinate bradsot cultures.
Rauschbrand serum did not agglutinate malignant-edema cultures.
Malignant-edema serum did not agglutinate Rauschbrand cultures.
Malignant-edema serum did not agglutinate pseudo-Rauschbrand cultures.
Rauschbrand serum agglutinated Rauschbrand cultures.

Grosso defines as pseudo-Rauschbrand a type of organism that resembles Rauschbrand closely and is pathogenic for mice; it does not form chains. Grosso isolated his organisms from the heart blood of guinea-pigs.

Markoff used the agglutination reaction to distinguish his strains which were of cattle origin. The filament-forming strains fell into one group and the nonfilament-forming ones into another.

Wulff (612) found the agglutination of cultures to be of value in distinguishing Rauschbrand from diseases that resembled it. The serum of cattle dead of Rauschbrand contains, however, no agglutinin.

Meyer (a, 458), made use of the agglutination reaction for the identification of his hog bacillus. The serum made with the hog bacillus agglutinated to a high titer a vibrion-septique strain of the Pasteur Institute and to only a low titer the blackleg and malignant-edema strains which were available.

McIntosh found that the agglutination reaction distinguished sharply between the groups of anaerobes. A B. chauvoei serum agglutinated its own strain to a high titer and a vibrion septique strain to a low titer, and the serum of the vibrion-septique strain agglutinated the homologous strain to a high titer and the B. chauvoei strain to a low titer.

The Anaerobe Committee recommend agglutination as a means of distinguishing the groups of organisms.

Robertson (b) found that the vibrion septique strains studied by her formed four groups on the basis of the agglutination reaction and she found that impure or recently isolated strains frequently failed to agglutinate. She considers a positive agglutination with a vibrion septique serum to be specific.

Zeissler (a, 110) asserts that he has found Rauschbrand 10 times as a human wound infection. Agglutination with a serum derived from a "Rauschbrand" (?) strain from a cow was the determinative measure used. (In 6 of these cases Fraenkel's bacillus was also present.) Zeissler does not say

whether the wound infections were gangrenous processes, or whether he isolated the organism merely from septic wounds. He says (b, 40) that the Ghon-Sachs bacillus so closely resembles the Rauschbrand bacillus that there is no fundamental or morphologic difference between the two, except that the former makes filaments in the animal body. As Zeissler worked with a colony method that should have distinguished true Rauschbrand from Ghon-Sachs-like organisms, I am forced to suspect that his "Rauschbrand" used for the manufacture of the agglutinating serum was an organism of the vibrion-septique group that showed little tendency to form filaments in the animal body, or perhaps that Zeissler did not look at smears from the peritoneal cavity to find filaments. This suspicion mounts almost to certainty because Zeissler found the bacillus of Ghon and Sachs only once in man and "Rauschbrand" 10 times.

Zeissler (a, 110) states that two anaerobe strains, which were manifestly of a different species from that with which a serum was made, were agglutinated by that serum. He guarded against auto-agglutination. He heartily condemns the agglutination reaction as a means of differentiation of anaerobes and calls into question all decisions that rest on it.

Passini finds that putrificus-immune serums may agglutinate organisms of another species to a higher titer than that to which they agglutinate other strains of putrificus. He intimates that this finding strengthens the thesis of Grassberger and Schattenfroh, namely, that the anaerobes are all more or less the same. I should suggest, instead, that the data of Passini show the worthlessness of the agglutination reaction, at least as practiced by him.

Robertson (private communication) immunized a rabbit with a strain of my own (B. II, Hempl) that is to be assigned to the bifermentans group. The serum of this rabbit agglutinated B. sporogenes. I do not think there is any reason to doubt the purity of the strain used by Miss Robertson.

My experience with agglutination in the differentiation of anaerobes has been distinctly unsatisfactory. I heartily agree with Zeissler in his condemnation of it. The phenomenon of auto-agglutination interferes frequently: in the oedematiens group it makes all agglutination work impossible; in the blackleg group it often gives trouble but may be eliminated by adjusting the electrolyte content of suspensions and by adjusting the P_H reaction; in the vibrion-septique group it causes little inconvenience. But there is a variable element in the reaction, existent in the cultures of the organisms to be agglutinated, which lies always ready to trap the unwary. I find that vibrion-septique strains fall into several groups on agglutination, as stated by Robertson (b); but when the experiment is repeated most of the strains will behave as they did before; one or two of them may behave, however, in so different a fashion that the worker is at first led to suspect that he has made mistakes in his technic, and is later forced to conclude that the medium and technic used are unsatisfactory for the problem in hand. The medium used by myself was a casein-digest broth containing a chunk of liver. All digest mediums have now been discarded and others are being tried. It is to be hoped that a technic and medium may be developed that will furnish regularly reproducible results. With a given serum some strains agglutinate to a high titer and some to a low titer. The phenomenon of an inhibition zone was also observed. Several strains are agglutinated by one serum while another serum agglutinates only one of them. One strain was agglutinated by serums of the three principal types. The agglutination reaction may in general be used to separate one group from another, but we have plenty of other criteria for such separation that are easier to use and more dependable.

The Medical Research Committee (39, 55) term the agglutination reaction "ultra specific." For the therapeutist this statement is probably correct, but for the systematist and for the epidemiologist the finer differentiations are of great value. It is to be hoped, therefore, that absorption-of-agglutinin tests may lead to some advance in breaking up the groups of anaerobes. Agglutination tests in the anaerobic group are not to be undertaken lightly with a few strains, and I do not believe that agglutination tests performed with impure cultures can be of value, at least not in the vibrion-septique and blackleg groups. It should be remembered that the use of another much-heralded immune reaction, namely phagocytosis, has been largely abandoned because of occasional inexplicable inconsistencies in its behavior.

(*g*) *The Precipitin Test.*—In large veterinary institutes in countries like Germany, where losses by Rauschbrand are recompensed by the government, the problem of determining the nature of such a disease cheaply and expeditiously from pieces of flesh sent to the laboratory is a serious one. Miessner and Lange propose the precipitin reaction as a solution for such a problem. They successfully immunized horses to Rauschbrand and to malignant edema. The precipitin reaction was carried out on the juices of the muscle of the animal in question. It failed or gave an equivocal result in 14 out of 65 cases that were anatomically and microscopically referable to Rauschbrand. But Miessner and Lange do not tell us what results these cases gave with a malignant-edema precipitin, nor do they give us other details as to the strains of organisms found in these cases. I think it very likely that the material that showed no precipitation with a Rauschbrand serum may have been from animals with an infection by an organism of another group, and thus worth investigating. One point must be suggested: Perhaps precipitin reactions on extracts from pieces of Rauschbrand muscle will work best for low virulent infections and less markedly for highly toxic infections because such reactions are probably dependent on the number of bacilli in the muscle. Miessner and Lange note great variation in the intensity of the reaction.

Ascoli and Valenti found the precipitin reaction of great use in the diagnosis of anthrax. Pfeiler says that anthrax is the disease par excellence whose diagnosis may be established by this reaction. He quotes several authorities who used it for medicolegal determinations on badly decayed cadavers that were entirely unsuited to microscopic or bacteriologic examination. He and Rehse obtained positive results with a sample of mud from a spot where a cow, sick of anthrax, had been slaughtered weeks before.

(*h*) *Hemolysins.*—Nicolle, Cesari, and Raphael showed that the hemolysins in 5-day Martin's broth cultures of their strains showed variations which were of no differential value. I find all organisms of the tissue-invading groups highly hemolytic in their action in blood broth.

(*i*) *Hemagglutinins.*—The same authors found that all these strains gave hemagglutinins for guinea-pig red blood cells, and that four of their B. chauvoei strains and one vibrion-septique strain gave hemagglutinins for rabbit red blood cells.

(*j*) *Leukocidins.*—Eisenberg found that a Rauschbrand antileukocidic serum was as effective against the leukocidin of B. oedematis maligni as against that of B. anthracis symptomatici. Eisenberg does not present details as to the identity of his strains.

(*k*) *The Complement-Fixation Test.*—Rocchi found complement fixation to distinguish anaerobes of various strains. Even different strains of the same name were not identified by this reaction. The collection was one of strains from various laboratories, and details as to their behavior are not given.

Ascoli and Valenti could find no application for the complement-fixation reaction in anthrax material.

(1) *Summary of Uses of Immunologic Tests.*—It may be concluded that for separation of the organisms of the blackleg group from those of the vibrion-septique group various immunologic methods may be employed. I should place these in order of preference with regard to convenience and value as follows: Immunization of guinea-pigs by serum, by toxin, by cultures; precipitation; agglutination. I should hesitate to classify the other methods.

I venture to say that immune reactions will in time automatically be relegated to the province of the serum laboratory where the antigenic status of the organism is its only interesting feature. We have or can develop abundant other methods for classifying the anaerobes according to their group affinities, and we have cultural methods that divide up the groups into smaller and more readily recognized subdivisions than do immune reactions.

II. An Investigation of Material Derived from Animals

By isolating organisms from animal material and from cultures derived from animals and by securing strains already isolated by others I have made a collection of 54 strains of pathogenic tissue-invading anaerobes. A classification of these strains according to their group affinities and according to their source is excerpted from the appendix.

1. PATHOGENIC STRAINS DERIVED FROM ANIMALS

Strains of the Vibrion-Septique Group 30: from cattle 13, from sheep 8, from horses 2 (one spontaneous infection, one accidental laboratory infection with a human strain), from hogs 2, from guinea-pigs 2, probably from guinea-pigs or rabbits 2, from whale 1.

Strains of the Blackleg Group 21: from cattle 19, typical, 15, atypical (probably none identical) 4, from sheep 2, one apparently typical and one atypical and unique.

Organisms of the Oedematiens Group, 3: from horses. Three edema producing organisms of the same species referable to the oedematiens group were specifically distinct from all other strains in my collection.

From a few specimens of original material from cattle and sheep I was unable to isolate pathogenic organisms—the material was probably too old. I much regretted this in the case of the sheep material from Professor Hamilton which was given me by Dr. Jensen, because Hamilton's material is in a way classic and has been studied by several workers. From a sadly large proportion of cultures it was found to be quite impossible to isolate a pathogenic organism. Proteolytic contaminations had completely outgrown the original pathogen. Still, a fairly large number of pathogenic organisms was recovered.

2. TECHNIC OF ISOLATION

The technic employed in isolation was, briefly, as follows:

Muscle material or culture received was inoculated into tubes of meat medium and was incubated anaerobically for about 20 hours. It was then examined. If the flora comprised aerobes, the culture was heated in a pipet and inoculated into fresh medium. If, on examination, the flora was apparently highly proteolytic, a large dose of 1.5-2 c c was injected into the thigh muscle of a guinea-pig; if the flora was nonproteolytic and suggested the presence of a known pathogen, a small dose of about 0.5 c c was injected into the thigh muscles of a guinea-pig. To avoid contamination of syringes by sporulating organisms, Pasteur pipets have been used for inoculations. This is the method used by von Hibler. Cultures were recovered from the heart blood after the death of the animal and were studied critically to detect contaminations. Further isolation was accomplished early in the period of the investigation by Barber's single bacillus technic (usually more than one bacillus from an apparently pure culture being used), and later by my usual deep colony method. All cultures were checked several times for purity by a deep colony method. When a culture with a history of pathogenicity was found to be nonpathogenic for a guinea-pig, it was inoculated (20-hour culture) into another guinea-pig with a small drop of lactic acid. If it then failed to infect, it was inoculated onto liver broth and the same technic was employed as for meat medium. To this type of perseverance I owe the unique and highly pathogenic strain AS. If no pathogenicity was shown after these efforts, the material was abandoned.

An interesting feature was that in three cases in which a mixed infection of a vibrion-septique type of organism and of a blackleg type of organism existed in the material from the original cow, as could be easily detected by the morphology of the organisms in the meat medium, a culture of the mixture on meat medium gave a vibrion-septique infection in the guinea-pig, and a culture on liver broth gave a blackleg infection in the guinea-pig. This could probably not be accomplished with all such mixtures, as vibrion-septique strains are known to differ considerably in pathogenicity. I know, however, of no other way, short of immunization of guinea-pigs, whereby blackleg may be isolated when a vibrion-septique strain is present.

This is an expeditious method for isolating pathogens. I do not pretend that I isolated all the pathogenic organisms in the material at hand. It is, however, probable that all the vibrion-septique strains present were isolated. I know of no cultural method of isolating blackleg from a gross mixture. Its colonies are so slow to develop that it would seem almost a hopeless task to try a colony method to isolate it unless it were in predominance. Vibrion-septique organisms may, however, easily be isolated from mixtures by a colony method.

3. DISCUSSION OF TECHNIC OF ISOLATION AND OF SOURCE OF MATERIAL

I am well aware of what some of my critics will say concerning this method of isolation. Foth emphasizes repeatedly that carefully dried muscle of animals dying from blackleg should be the material used in inoculating the guinea-pigs by whose aid the primary isolation is made. Otherwise, says he, contaminating organisms may also infect the guinea-pig. Contaminations are of three kinds: nonsporulating organisms, which I always kill by heat before the culture used for inoculation is grown; sporulating organisms capable of penetrating tissue in the company of another organism only; and sporulating organisms of high

pathogenicity. The two latter groups Foth lumps in general together as "verbandbildende Bakterien." If one has only one guinea-pig to use per strain it would be unwise to give it a heavy dose of mixed anaerobic culture because proteolytic forms might reach the heart blood and the culture therefrom be impure. But may I assure the reader that the element of proteolytic contamination is one with which I am so thoroughly familiar that I should never fail to recognize it and to purify further any culture in case it existed. It would be impossible for me to mistake a mixed blackleg-proteolytic infection or a culture from such an infection for "malignant edema." I think that a great many mistakes may be traced to the fact that the blood of an infected animal usually does not contain enough organisms for the inoculation of animals or even for reliable colony work, and therefore transudates have been used for inoculation purposes. But if one take the heart blood of an animal dead from a blackleg infection or an infection by a member of the vibrion-septique group, inoculate it into a tube of meat medium, and incubate the tube anaerobically over night, one will invariably secure the principal invader, usually, but not always, in pure culture. It never pays to use subcutaneous serosity or peritoneal or pleural transudate for cultural purposes. One should beware of the fact that certain other types of anaerobes, notably B. oedematiens and B. Novyi, frequently kill guinea-pigs without becoming septicemic, and must be obtained from invaded muscle or from edema fluid; from undiagnosed infections, therefore, cultures should be made from various regions.

The critic will say, however, that, given a mixed culture of blackleg, vibrion septique, and a proteolytic organism, I might eliminate quite satisfactorily the proteolytic organism and lose also the blackleg strain because of the presence of the more highly pathogenic strain of the vibrion-septique group. I confess that this might happen and that it may have happened in the series of strains here presented. But if the blackleg strain and the vibrion-septique strain were present in abundance, as they usually are in material from an animal that they have killed, I should emphatically assert that they would both easily be detected in the guinea-pig, pathologically and microscopically. I know my critics will say that this is impossible, but not only am I entirely confident that it is possible, but I have three cases to prove it. I should remark first, that no culture ever passes my hands for inoculation purposes without microscopic examination of a Gram stain made from it; second, that careful necropsy examinations are made of all animals, their lesions noted, cultures taken from the heart blood and from infected muscle far from the site of inoculation, and smears or impressions made from the site of inoculation, inguinal region, axilla, peritoneal wall, liver surface, and from organs with unusual lesions. These smears are stained by Gram's method and carefully studied by myself. I have so studied every animal—over 200 in number—that has died of an anaerobic infection in this laboratory, and I should decline to accept the criticisms of one who was not in the habit of making like careful observations. Four times in the study of my material from cattle I have thought blackleg and an organism of the vibrion-septique group to be present in a guinea-pig infection. Three times I succeeded in isolating two strains—a blackleg strain and a strain of the vibrion-septique type—from the material. Each time I had thought blackleg to be present when examining the original culture which was used for inoculation. The fourth time a vibrion-septique strain alone was present (strain AS from a badly contaminated culture). It is an organism that forms spores lying sidewise in the bacillus and it forms large spoon-shaped orgonts; but otherwise it is to be assigned to the vibrion-septique group. I should hold that, had a cow died of a mixed vibrion-septique-

blackleg infection, and were the two organisms present in anything like equal proportions in the material given me, I should hardly fail to detect both infections.

But my critics will say: "Suppose a cow died of blackleg and an organism of the vibrion-septique group contaminated the material—would you then not be likely to assign your case to mixed infection when the infection was truly monospecific?" The critics would be right. But the interpretation to be made of such a possibility should be on the basis of a true understanding of the chances of such a coincidence. It would probably be somewhat more likely that a vibrion-septique strain might contaminate blackleg material than that blackleg should contaminate vibrion-septique material. But the members of the vibrion-septique group are by no means ubiquitous in their distribution. Carl (c. 869) reviews this subject. Cornevin found the vibrion-septique type of organism rare in forest earth. Jensen found malignant edema rare in earth from various sources in the vicinity of Copenhagen. Feser infected only 7 of 30 rabbits inoculated with earth from various filthy places; among his samples was earth from the vicinity of anthrax cadavers. He does not tell us how many of his invasions were real infections of the vibrion-septique type. Carl concludes that the "malignant edema bacillus," under which name probably are included strains which are not of the vibrion-septique group, is widely distributed, but that it must not be regarded as a ubiquitous organism. We must also remember that we have no proof that the blackleg type of organism is not almost as abundant in distribution as are the organisms of the vibrion-septique group. The blackleg organisms are not pathogenic for many animals, but both types are highly pathogenic for cattle, and cattle are frequently attacked by blackleg. It is not even proved that blackleg organisms are regional in their distribution because blackleg is usually a regional disease. It is more than probable that some factor other than the mere presence of the organism induces infection, and it may be that such factors, rather than the bacillus, are regional in occurrence.

Another point indicates that my strains are not to be taken as grossly misrepresenting the flora of cattle and sheep infections. Guinea-pigs are subject to invasion by various soil organisms; yet I have found no strain from cattle material which was capable of invading guinea-pig tissue on its own initiative which does not fall into one of the two classic groups of cattle invaders. The nonsporulating tissue invaders are, I admit, to some extent eliminated by my technic, but nevertheless, had specifically abundant and gross contaminations of my material taken place before it reached me, other organisms would have been found, as one finds them any day in soil, which would have been pathogenic for guinea-pigs.

As to the material which came into my hands in the form of cultures: For the original mode of isolation of this I can take no responsibility. I place particular importance on that derived from cattle because it was called blackleg by someone else, presumably by the veterinarian who diagnosed the case in the original animal or by the bacteriologist who first studied the organism. It is possible that in one or two of my cases an original blackleg may have been supplanted by a vibrion-septique organism, but to my mind it is improbable that it happened in many cases.

4. STRAINS ISOLATED FROM CATTLE MATERIAL

From cattle material there were isolated 19 strains of anaerobes that are referable to the blackleg group and 13 strains referable to the vibrion-septique group. Three mixed blackleg-vibrion-septique infections were recognized.

Though I regard these findings as of great importance, I do not wish to be understood to present these figures as exactly representative of the relative frequency of these infections in cattle. There are several points that make them ineligible as statistics.

First, they are insufficient in number and they represent scattering cases from the United States and a few from Europe.

Second, their history is varied and inaccessible to me.

Third, some of them have been selectively weeded out by persons cognizant of the difference between blackleg and vibrion-septique infection—Eichorn's strains which he used for vaccine, for example, were all five referable to the blackleg group.

Fourth, a worker would be somewhat more likely to fail to recognize the presence of a blackleg strain than that of a vibrion-septique strain in an old and partially run out culture, because B. chauvoei is a less active grower and less pathogenic for the guinea-pig than the organisms of the vibrion-septique group. Likewise, blackleg would probably more frequently be entirely lost from cultures because of its delicacy.

Fifth, there is probably a greater chance of contamination of cultures and material by vibrion-septique organisms than by organisms of the blackleg group. This element, though it exists, is not, to my mind, of great importance. I hold that such contamination may have taken place in my material, but that it is unlikely that it often did so. A prime point of evidence in support of this opinion is the fact that the four mixed infections (one from sheep), recognized by me, came from original animal material, and the fact that less than a third of the older cultures contained a pathogen. Were contaminations an important source of vibrion-septique strains, the older cultures would contain a large number of such strains.

The following table may be of interest:

TABLE 2

Strains Isolated from the Cattle Material Studied

	Animal Material, 14 Samples	Brain Cultures, 7 Samples	Agar or Gelatin Cultures, 23 Samples
2 pathogens isolated	3	0	0
Blackleg group	3		
Vibrion-septique group	3		
1 pathogen isolated	11	7	7
Blackleg group	11	3	2
Vibrion-septique group	0	4	5
No pathogen isolated	0	0	16

The agar and gelatin cultures may in general be taken to have passed through more inoculations and generations than the brain cultures, which were handled by workers more familiar with anaerobes than those who used agar and gelatin cultures. It will be noted that the handling and transplanting of anaerobe cultures, a process which tends always to promote contamination of such cultures, tends also greatly to weed out pathogens and to replace them with nonpathogens. This finding is fully substantiated by a study of human material and cultures from human material. The pathogens may be regarded as rare and as infrequent contaminators of other material. I have only once discovered a vibrion-septique strain as a contaminator of a culture when there

was no record of the presence of an invading pathogen. The culture was a stock tetanus strain of whose previous history I am ignorant. I do not think that loss of virulence is to be considered in connection with these figures. Vibrion-septique strains apparently do not lose virulence; they are among the most highly pathogenic of organisms. It will be noted that strains of the blackleg group preponderate greatly over the vibrion-septique strains in original material from cattle. This might be taken as an indication of contamination of cultures by vibrion-septique strains when they are subject to laboratory processes; but it must be borne in mind that the majority of the muscle samples received were from laboratories that used the strains for the making of vaccines, and they thus were subject to a deliberate weeding-out process. That in old cultures vibrion septique should be the most frequent form may or may not be wholly due to the fact that B. chauvoei is more delicate and more easily lost.

I must, however, say frankly that I should hesitate to conclude from the above figures and to assert emphatically that vibrion-septique infections were common in cattle, were it not for the fact that I feel that the accounts in the literature support me in such a conclusion, and that the close analogy between the infections of cattle and those of horses and sheep and hogs points to this probability. Countless evidences in the literature bear me out in my contention that strains of the vibrion-septique group are frequently isolated and identified as blackleg. Again and again one finds them—many have been quoted in this paper and many more will be observed on more careful search. Examples of these mistakes may be found with like frequency in various countries, and the errors have been made both by pathologists and by trained bacteriologists. They were made many years ago and they are made today. Only a few workers, all of them men who have themselves handled numbers of strains, have seen the light on this subject. Some of them worked when bacteriology was young and some have worked more recently. They express themselves, their work becomes classic, and then it is promptly disregarded in its principal points by the rank and file.

I feel justified in charging the majority of the members of the veterinary and bacteriologic professions who have dealt with the subject, with having misunderstood the etiology of the anaerobic invasions of cattle. They must either give up the habit of assuming that the lesions hitherto diagnosed as "blackleg," or considered as characteristic of blackleg, may be so diagnosed without differential bacteriologic examination; or they must completely relinquish, in connection with the conceptions "Rauschbrand," "symptomatic anthrax," and "blackleg," any idea that these names describe a disease sui generis, and must deliberately include under these names infections by the members of the vibrion-septique group.

5. STRAINS ISOLATED FROM SHEEP MATERIAL

I have isolated from sheep material two strains of organisms that are referable to the blackleg group. Both appear to have been the only pathogen present in their respective specimens. One, a strain apparently resembling in all respects the majority of those from cattle, was isolated from sheep material sent me by Dr. Hadleigh Marsh of Montana. He had diagnosed the case as blackleg. The other, from Halle, Germany, was derived from bradsot material sent me by Dr. Jensen; it is definitely referable to the blackleg group, but in morphology and in colony formation it differs from all the other strains under observation.

How much light my investigation of bradsot material will throw on the subject of sheep infections I do not know. The material studied was all from

cases that had been diagnosed as "bradsot" by different workers in different countries. It is therefore parallel to my cattle material which had all at some time been diagnosed as "blackleg." I have to thank Dr. C. O. Jensen of Copenhagen for all the samples studied. They were, with one exception, pieces of muscle from what I understand to have been the original sheep. There were 11 samples: from 3 I could isolate no pathogen, from 1 the blackleg-group strain RG was isolated, and from 7 samples strains of the vibrion-septique group were isolated. One specimen from Sletten, Norway, furnished two species of vibrion-septique type. They differ markedly in colony formation and in pathogenic action on the guinea-pig, one producing on thigh inoculation the most marked gastritis observed in this laboratory, with a bright red coloration over the whole external stomach surface; the other producing a relatively small amount of injection in the abdominal cavity. These 8 strains are all definitely to be included in the vibrion-septique group as defined on page 389, b. They are by no means all of the same species, but show on careful study various differential characters. A definition of these characters is forthcoming. If these strains are to be accepted as the etiologic agents in cases of bradsot, then we must say that bradsot is not a disease sui generis.

I realize fully that my lack of case histories must qualify any statement as to the causative rôle that these organisms played in the death of the sheep from which they came. The final criteria to be used in judging of what is braxy and what is not braxy, of what constitutes anaerobic infection and of what must be ruled out as not constituting anaerobic infection are, to my mind, still to be established. But the fundamental bases for such criteria should be etiologic and histopathologic ones.

The fact that pathogenic anaerobes may be found in animals dying of other causes is, to my mind, entirely beside the question. Careful and intelligent determination of the quantity and specific nature of the anaerobic invaders in fresh cadavers of sheep showing "bradsot" lesions and in fresh cadavers of sheep dying of other causes is the only way to make observations that can be considered of great value.

Data concerning other strains isolated will be found in the next part of this article.

A note as to the subdivision of the groups discussed is perhaps in order. Various authors have noted differences in anaerobes which had been classified as one species by other workers. Many of these differentiations depended on culture-contaminations. Under this head I should classify Titze and Weichel's strains, Markoff's proteolytic and nonproteolytic chain-forming strains, and Klose's strains. Some divisions depended on an association and further division of unrelated organisms. Kirsten investigated many carefully-isolated strains of what he calls the edema bacillus. He found the organisms in feces and in the blood of various animals. Most of them were nonpathogenic. Kirsten describes 10 types which can be distinguished by morphology and by cultural characters. I interpret these strains to have been members of various saprophytic and parasitic groups. Some differentiations have been made whose value I am unable to determine. Poels, whose original article is inaccessible to me, cultivated blackleg bacilli from what I take to have been the inoculated thigh of guinea-pigs. He noted differences in the gas formation, in the time of sporulation, and in the pathogenicity of his strains. Grosso divides his blackleg strains into "Rauschbrand"—nonpathogenic for mice—and "Pseudo-rauschbrand"—pathogenic for mice. Robertson (Med. Res. Comm. 39, 53) has divided vibrion septique into 3 groups on the basis of the agglutination reaction. Weinberg and Séguin (b) note minor differences in their vibrion-septique strains.

A few details as to the differences between the strains in my collection must be included. Most of the blackleg strains behave alike, so far as they have been studied. These I call temporarily "typical" blackleg. They form heavy citron-shaped orgonts, are quite highly pathogenic for guinea-pigs and are not pathogenic for rabbits. Their colonies are similar. A few other strains behave differently. One is different in morphology and is only slightly pathogenic for guinea-pigs. Another has never been observed to form citron-shaped orgonts, another forms colonies that are unique. A careful investigation of other characters of these organisms is in progress. Until this investigation is finished I must decline to divide the group in a dogmatic manner. The strains of the vibrion-septique group are divisible into subgroups by agglutination, by colony formation, and to some extent by morphology. I must also decline to arrange these divisions for the present.

III. The Anaerobic Infections of Animals as Recorded in the Literature: A Critical Analysis of the Anaerobic Infections of Cattle

(*a*) *The History of Cattle Infections Up to* 1905.—This article deals with the organisms involved in the anaerobic infections of animals. I am in no way prepared to discuss with authority the subject of the pathology of the disease of blackleg in cattle, and must be content to refer the reader to some of the notable works on that subject. It will be convenient to discuss simultaneously the two types of organisms and the diseases caused by them.

Anthrax and symptomatic anthrax (blackleg) are two diseases that are in general so similar that ancient and medieval authors do not distinguish them. According to Arloing, Cornevin, and Thomas, who have most carefully reviewed the literature from ancient times on, a satisfactory and logical classification of anthracoid diseases was not made till Chabert undertook to arrange them according to clinical symptoms in 1782. He classified them thus:

Fievre charbonneuse: "When the disease develops without manifesting its existence by external tumors, it is anthrax fever or internal anthrax."

When tumors appear, it is "essentiel" or "symptomatique."

Charbon essentiel: "It is "essentiel," when the tumor begins at the onset of the disease without former symptoms and without signs of sickness except those which result from its existence, the tumor being at first small, hard, renitent, painful; then enlarging, and only after enlarging producing grave constitutional symptoms. When incised these tissues are black, like gangrenous tissues."

Charbon symptomatique: "It is symptomatic, when the tumor follows a rise of temperature, loss of appetite, arrest of digestion, chills, and rigidity."

Feser is given credit for being the first to distinguish Rauschbrand from anthrax in recent times (1876).

Bollinger was the next to publish material on the subject (1878).

Arloing, Cornevin, and Thomas, in 1879 and following years published a series of papers concerning the disease, which afterward were condensed in book form under the title "le charbon symptomatique du boeuf" and appeared in two editions, the latter in 1887. They reviewed all the literature available on the subject and published accounts of their own valuable researches.

(*b*) *Vibrion Septique and Atypical Blackleg Group Infections of Cattle.*—Malignant edema has often been reported in cattle. I have neglected to collate as long a list of such reports as I might have done, and I am ignorant of the period in which such infections were first recognized.

Puerperal blackleg has often been discussed. Dr. Meyer informs me that some cases of so-called puerperal blackleg are not due to anaerobic invasion. Many cases have, however, been reported as due to organisms of the vibrion septique group.

Some reports of unusual blackleg infections cannot be accurately placed.

As stated before, I am in no position to express an opinion on the pathology of these diseases in cattle. I think that certain suggestions are in harmony with those of Foth and others, and that on the analogy of the behavior of these diseases in other animals certain lesions will be found characteristic in cattle. Blackleg organisms show in general a preference for skeletal and heart muscle, and they produce a greater amount of hemolysis and a smaller quantity of edema, gas, and transudate than do those of the vibrion septique group. Gas is probably most frequently in the form of minute bubbles in the muscles themselves. The organisms of the vibrion septique group grow well enough in muscle but will probably be found to kill, in nature, most often before muscle invasion is extensive. They prefer areolar tissue and mucous membranes and serous linings to muscle. Muscle lesions when present probably do not show the dry, spongy, almost brittle character of blackleg muscle lesions and may be markedly infiltrated. The organisms of the vibrion septique group produce abundant gas in

areolar tissue and in muscle, where it is in larger bubbles than in blackleg lesions, and where it is interstitial in its distribution, and they produce little or no gas in the mucosa. The type of infection, apparently rare, in which the mucosa of the alimentary canal are markedly injected may well be termed "braxy" or "bradsot" of cattle.

This should be emphasized: When a worker with suspected blackleg material from a cow finds both true blackleg and an organism of the vibrion septique group present in the affected muscle of that cow, he has no right to conclude that the organism of the blackleg group alone was the cause of death of the cow; and a worker who finds an organism of the vibrion septique group and no organism of the blackleg group in the affected muscle of a cow dead of a blackleg-like disease is as justified in pronouncing such a strain of the vibrion septique group to be the cause of the death of the cow as he would be justified in pronouncing the disease "blackleg," were the organism of the blackleg group. That organisms of the vibrion septique group are pathogenic for cattle needs today no further proof. Why then persist in overlooking such infections?

(*c*) *An Oedematiens Group Infection in a Cow.*—Kerry describes an organism of oedematiens type which he isolated from a cow that was thought to have died of blackleg. The organism, according to its description, resembles the B. oedematis maligni II, described by Novy in 1894 and isolated by him from a guinea-pig that had been inoculated with milk nuclein. Kerry did not work with Novy, as has often been stated. Whether exact cultural comparison of the two strains was ever made I am unable to determine. Kruse identifies the two strains and names them B. oedematis thermophilus Novy. Migula renamed the species B. Novyi, which name is probably the least likely to lead to confusion. As identifications by means of printed descriptions are unsound, we must let the specific affinities of Kerry's organism go undefined until a strain can be compared with that of Novy and with those of Weinberg. Kerry's organism is far more pathogenic for guinea-pigs than any of the strains of B. Novyi in my possession, and the lesions that it produces (Kitt, e) resemble more closely those produced by the oedematiens strain Joly.

(d) The Epidemiology of Anaerobic Cattle Infections.—Under this above title must be considered the contributory mechanic-traumatic factors leading to infection.

Blackleg as a Wound Infection: Most older authorities agree that blackleg is a wound infection, although frequently the wound may not be large enough to be demonstrated. There are many accounts of necropsies in which no external lesion was found. Therefore many authors have concluded that infection probably often occurs through the alimentary canal.

Blackleg as an Alimentary Canal Infection: The idea permeates the literature that malignant edema is a wound infection due to much graver wounds than those to which blackleg is due. Textbooks all give the disease as a wound infection, never mentioning possible infection through the alimentary canal, though it is now recognized that braxy may infect sheep in this manner, at least occasionally (see p. 431).

I conceive the broad epidemiologic factors in these diseases to be as follows: In the soil in general, wherever life processes occur to any appreciable extent, organisms of the anaerobic group are to be found. The vast majority of these organisms are probably not truly parasitic at all. Different species possess different powers of tissue penetration. To some extent different animals possess different defensive powers against the various species of anaerobes. In general the most highly pathogenic species of anaerobes are comparatively rare in their distribution and wounds do not become heavily contaminated by them and do not frequently become infected. But in regions where animals have died of anaerobic infection since time immemorial there occurs such heavy contamination of wounds by anaerobic organisms that such wounds frequently become infected, and animals ingest quantities of pathogenic organisms, and the chances for infection are multiplied, and whatever be the factors promoting infection, animals occasionally develop the disease, and the district becomes known as a "Rauschbranddistrikt."

That valleys and meadows and swamps should be the most dangerous places is easily understood, when one considers that they are the most closely grazed, that valleys are natural highroads, and that mud is a very active agent in the promotion of wound infections. Then, too, it must be remembered that the disease is one of summer time, in other words, of the grazing country. Doubtless farmers are more careful to destroy carcasses of animals dying near the stables.

To what extent excessive infection of the soil is due to other factors than the contamination of the soil by dead animals, probably no one is at present prepared to state. Le Clainche and Vallée showed that blackleg organisms were present in the feces of cattle which inhabited "blackleg districts." The carelessness of stockmen in the disposal of carcasses is certainly a factor in the spread of these diseases. District veterinary Kjoss-Hanssen of Egersund, Norway, states that as soon as sheep are known to have bradsot the peasants slaughter them, the bodies are butchered, the intestines are thrown to the wind and weather or at most buried in mounds, wherefore contagion is spread over the fields as in the old days. Hamilton, (a, 292) speaking of the Scotch shepherds, says that if the body of a braxy sheep is so decomposed that it is unfit for food it is left in the fields after skinning, or may be thrown on the manure heap. Marsh speaks of a farmer in Wyoming who let 20 carcasses of sheep, dead of blackleg, lie long in a field. Wulff (673) says: "Bis vor wenigen Jahren wurde diese Krankheit (Geburtsrauschbrand) allgemein dem echten Rauschbrande

zugezählt, und noch heute vertritt mancher Praktiker (Honecker) den Standpunkt, dass diejenigen Fälle, die nicht als puerperales malignes Oedem positiv erkannt werden, als Rauschbrandverdachtsfälle wie Rauschbrand veterinärpolizeilich zu behandeln und zu entschädigen sind." It appears to me that Wulff is entirely wrong in implying that there is no need of destroying bodies of animals dead of malignant edema infections. His point of view is directly traceable to the oft quoted idea that the bacillus of malignant edema is universal in its distribution and contaminates every open wound. This in turn is due to the contamination of cultures of nonproteolytic pathogens with the universal proteolytic forms. I should suggest that it were a greater crime against society to leave unburied or undestroyed a cadaver dead of a vibrion septique infection than one dead of a blackleg infection—the organisms of the vibrion septique group have a far greater range of possible hosts among domestic animals, and they are probably, in many cases, at least, quite as pathogenic for cattle as are blackleg organisms. Such laws as exist concerning the destruction of blackleg carcasses should most certainly be construed to cover carcasses of animals dead of both types of infection.

It is conceivable that one type of anaerobe may be responsible for most of the infections of a given district. Such a point should, however, never be taken for granted in any one case. It is also conceivable that one type—perhaps the blackleg type—may infect through the alimentary canal or by some unknown means with more facility than do the organisms of the other type.

It is evident from this discussion that the mode of infection in blackleg, beyond its being an occasional contamination of gross wounds, is entirely unknown. Most discussions as to the portal of entry of the blackleg bacillus revolve around wound infection of some sort, external or alimentary. Because of the fact that blackleg organisms do set up local processes where they are inoculated, and because in braxy the site of inoculation and the site of maximum proliferation is apparently in the alimentary canal, it seems highly improbable that blackleg is frequently a primary alimentary canal infection. It appears to me that the possibility of insect transmission of blackleg has been seriously neglected.

Insect Transmission of Blackleg and Allied Diseases: I have discovered no reference in any textbook to the rôle that may possibly be played by insects in blackleg infection. The idea of such a mode of infection is, however, an old one.

Hintermeyer describes an epidemic of quarter-evil, which raged in the summer and autumn of 1846 among the deer of the park of Duttstein, which Budd quotes somewhat inaccurately: "Als wesentlich habe iche hier noch zu bemerken, dass die Bremsen, und zwar (a) die grosse Rind-bremse (Tabanus bovinus), (b) die Regen-bremse (Tabanus pluvialis), und (c) die Blind-fliege (Tabanus coecutiens) welche im verflossene Sommer in unzähliger Menge vorhanden waren, wohl mit Recht als die theilweisen Träger des Contagiums anzusehen sind, und daher einere grössere Ausdehnung der Seuche verursachten. Diese setzten sich gewöhnlich zu Tausenden, auf die Cadaver der gefallenen Thiere, saugten die aus Maul, Nase, und After kommenden Profluvien ein, verliessen sodann die Leichen, begaben sich sofort auf gesunde Stücke stachen ihren von Contagium Saugrüssel in die Oberfläche der Haut ein, und inoculierten auf solche Weise das Seuchengift." We cannot be sure, of course, of the identity of this epidemic.

Guillebeau suggests that the larvae of bot flies (Hypoderma bovis) may cause infection of cattle in their wanderings from the alimentary canal to the skeletal muscle and to the skin; they make minute canals through the flesh. I

have found no confirmation of this suggestion in necropsy reports. Eggimann states that there is no Rauschbrand in Emmentahl and there are few warbles in hides from there, while skins of cattle from other valleys contain warbles.

I have found only one reference to experimental work in insect transmission of blackleg. Sauer caught common flies and biting flies which had passed a Rauschbrand cadaver. A small number of heads of these animals sufficed to produce typical Rauschbrand in guinea-pigs when inoculated subcutaneously. The bacilli were shown to multiply in the bodies of the flies, but the newer generation were smaller and weaker. Flies which had been on Rauschbrand cadavers were placed with wounded guinea-pigs, and they infected them in two cases. It was not shown whether the bites of flies could infect guinea-pigs.

A Laboratory Infection by an Organism of the Vibrion Septique Group.—I think the possibility of anaerobic infection by a small number of organisms entering through a minute wound, as would be the case in insect inoculation, has been overlooked. In this connection I may state that I recently infected my forefinger at the distal segment with a few washed organisms of a 24-hour culture of strain AS, of the vibrion septique group. The wound was a minute prick with a fine capillary pipet, and it was not more than 2 mm. deep. The finger became painful in 5 hours and swelled slightly, but was not feverish nor did it become cooler than the others. It was carefully kept warm to foster an active circulation. Twenty-four hours after the inoculation, however, on account of neglect, the finger became chilled and it immediately swelled perceptibly. Two hours later it was immersed in hot water and the swelling went down somewhat. Pain and swelling remained in the distal segment of the finger for a month, though fever and a tendency to suppuration were not noticeable. Apparently the infection lingered in the synovial membranes or in the tendon sheath. This is a case of invasion by an organism of the vibrion septique group in which no necrotic tissue was present, in which the size of the wound was infinitesimal, in which the depth of the wound was slight, in which no foreign débris was introduced, and in which no appreciable amount of culture toxin accompanied the organisms. But it will be noted that the organisms introduced were in the vegetative state and had been actively multiplying shortly before they were inoculated. I am confident that had circulation for some reason been partly suspended in the infected finger, the results would have been serious. I have found no other record of a laboratory infection by anaerobic invaders.

Perhaps it will not be considered mal apropos if I also introduce a few references as to transmission of anthrax by insects.

Virchow says: "Most commonly insects with piercing probosces effect the inoculation, such as gadflies (bremse); but flies which make no wound may also implant the poison on the skin by their soiled wings and feet."

Bourgeois expresses himself to the same effect.

Budd (1863) gives various points indicating insect transmission of anthrax. All but one of the cases of malignant pustule of the face which he has seen occurred in hot weather, in summer and autumn, while malignant pustule of the hands occurs all the year around. He cites two cases of women who were bitten by flies and developed malignant pustule at the site of the puncture, and another case which was caused by the puncture of a gadfly that came out of a fleece of wool.

Raimbert concludes (1869) from certain experiments that anthrax may be transmitted by flies, and that it is more probable that it is transmitted by flies that feed from cadavers than from flies that live by bloodsucking only.

Koch states that practically all the anthrax of the Russian steppes occurring at harvest time is due to insects, but does not give proof of his statement.

Minzmain (1914) successfully infected guinea-pigs with anthrax by means of the biting flies Stomoxys calcitrans and Tabanus striatus. The flies were applied to the hosts shortly before the death of the latter, and immediately after or 10 minutes later were applied to healthy guinea-pigs. The exposed animals died during the evening of the third day of typical anthrax.

Morris infected wounded guinea-pigs with anthrax by means of nonbiting flies.

As far as I can make out there is a generally accepted opinion among entomologists and veterinary scientists that anthrax may be, and frequently is, transmitted by flies.

The nature of blackleg is not so different from that of anthrax. The anaerobic organisms in the vegetative form are easily killed by air, as I have found in using the Barber technic of isolation. It is possible that inoculation of spores even in small numbers may prove infectious, but it is my opinion that inoculation of vegetative rods is much more so. If the statements of Sauer are correct, we may conclude that vegetative forms of the bacillus may be found in the bodies of flies. It is my opinion that insect transmission of blackleg is exceedingly likely. When one sees the charts representing the incidence of blackleg in "Le charbon symptomatique du boeuf"; how the malady increases in May and reaches its zenith in July, decreasing and practically disappearing in some years in September and in others in October, one is strongly inclined to look no further for a means of transmission of the disease. But it must be borne in mind that summer is the time of abundant grass in Switzerland. In California, Dr. Traum assures me, blackleg is to be expected whenever the grass is abundant, in fall or spring as the case may be. In Switzerland (Arloing, Cornevin, and Thomas) anthrax and blackleg occur at the same time; in California anthrax occurs in summer and fall when the ground is bare. It has frequently been noted that the fattest cattle die of blackleg, the finest sheep die of braxy. The explanation has been sought in the greater abundance of sugar in the muscles of animals that are well conditioned. Möller states that guinea-pigs that are sugar fed show much gas on blackleg inoculation, those that are not sugar fed show less, those that are starved show none at all. Guinea-pigs that are starved are less likely to succumb to blackleg than those that are well fed. I should say that this element must be thought of as a predisposing factor and not as a factor leading to infection.

Farmers in some portions of California often attribute blackleg infection to the drinking of contaminated water. Farmers of other districts in which the water is excellent are emphatic in asserting that the infection is in the food. There is not necessarily a single avenue of infection in these diseases, no more than there is in anthrax, and the demonstration of one mode of infection does not prove that it is the only or usual mode.

One strong point opposing the view that vibrion-septique infections are not rare in cattle is the apparently comparatively satisfactory protection furnished cattle by vaccination. Kitt (a) states that in Bavaria between the years 1898 and 1907, 0.144-0.320% of vaccinated cattle died of blackleg and 0.551-2.497% of unvaccinated cattle died of blackleg. Balavoine's figures for Switzerland are not very different from Kitt's. Blackleg aggressin is said to protect cattle even better than the bacterial vaccines. To what extent vibrion septique strains have been unintentionally used for vaccination purposes it is at present impossible to estimate.

The epidemiology of these infections is one of the most interesting in human and animal pathology. I regard it as an almost virgin field for investigation. It should be studied, and studied exhaustively, not by the veterinary pathologist, but by the trained anaerobist, who will investigate thoroughly every case that comes under his observation, and will not be satisfied that the first organism that comes to hand is the only one involved in the death of the animal before him, until he has looked carefully for others. Under the investigation of the epidemiology of blackleg and malignant edema should come the investigation of the immunity of older animals to these diseases, the immunity of animals from districts free of blackleg and braxy, the relative immunity of cattle and sheep of different breeds, infection of wounds and infection through the alimentary canal, infection by means of insects, and infection through causes that are now unknown to us—innumerable problems could be suggested. We shall not, however, be in a position to make such problems a profitable study until more is known as to the specific members of the groups involved.

(c) Conclusions as to the Anaerobic Infections of Cattle.—The conclusions as to the anaerobic infections of cattle which I derive from study of the above mentioned material and from a study of the literature of the subject of animal infections are:

Theoretical considerations lead one to conclude that cattle infection by a single species of anaerobe may take place, and that this species may be an organism of the vibrion septique group, of the blackleg group, or of the oedematiens group; or a mixed infection may take place by members of the different groups or by two members of the same group, or some other combination of organisms may invade the tissue. I find no record of B. Welchii infection in cattle. There is probably no reason why it may not be found as a wound infection, nor why other organisms may not be found, nor why proteolytic organisms may not also occasionally invade bovine tissue with the non-proteolytic invaders as they are known to do in man and small animals. Probably some of the ideas concerning the proteolytic nature of malignant edema are due to the observation of such cases.

Practical considerations lead to the following conclusions: Cattle are frequently subject to spontaneous infection by members of two groups of anaerobic invaders, the blackleg group and the vibrion-septique group. Both types of these infections are, in the vast majority of cases, diagnosed as "blackleg," "Rauschbrand," or "symptomatic anthrax." Infection by members of the first group is commoner than that by members of the second group, but infection by members of the second group is by no means to be ignored and probably should be considered in cattle immunization.

It is possible that a member of one group may be the predominant invader in one district, and a member of the other in another, which indicates the necessity of determining the bacterial agents involved in "blackleg" infection in various districts.

It is also possible and, to my mind, probable, that factors exist that induce anaerobic infection in cattle, whose identity it is important to solve. Beyond the knowledge that these infections may follow wound contamination we know nothing positive as to the mechanism of anaerobic infection in cattle.

Careful, intelligent, and thorough bacteriologic investigation of fresh cadavers of many animals dying of spontaneous "blackleg" infection is much needed, and connected with it is the need of an exhaustive epidemiologic survey of the field. The attitude of the veterinary profession has been that we already possess all the necessary knowledge concerning blackleg. It appears to me that we are in the morning of the investigation of blackleg and allied diseases, and that what we today know is but an inkling of the true state of affairs. An investigation of the subject that will properly correlate and solve the problems involved can be undertaken only after many workers have made studies of many cases from different districts. The reasons why this has not already been done are two: anaerobic technic has not been adequate for such study, and the idea of the monospecificity of these invasions has tenaciously held the field.

(f) The Immunization of Cattle.—It is not my purpose here to enter into a discussion of the extensive subject of the immunization of cattle against blackleg by bacterial products.

Arloing, Cornevin, and Thomas were the pioneers in immunization by killed and attenuated virus. Kitt modified their technic and also introduced serum virus inoculation for the production of immediate protection. His article in Kolle and Wassermann's Handbuch (IV, 1913) may be referred to as a modern authority on the subject. The use of filtrates for immunization of cattle was first suggested by Roux in 1888 (b) on the basis of protection afforded to laboratory animals by filtrates of infected guinea-pig tissue. Today various means of immunization are in use. Injection of powdered attenuated muscle virus and of pellet vaccines is still widely practiced, and tissue and culture filtrates, the so-called "aggressins," are apparently gaining in favor because of the safety attendant on their inoculation. Serum immunization is used for herds in which blackleg has recently occurred.

Suffice it to say that immunization of cattle against blackleg is regarded as highly necessary in some districts and as advisable in others. But it must be emphasized that such immunization is not always successful and that inoculation infection ("Impfrauschbrand") is not uncommon. These facts fit in so plausibly with my own figures and with the thesis of this paper that I am forced to conclude that the subject of the immunization of cattle deserves a careful investigation.

The only case of inoculation blackleg that has come to my attention is that of Dr. Haslam (private communication). The causative organism was a member of the vibrion septique group of the general type of the strain called AS. The spores sometimes lie sidewise in the bacillus and this type of organism is one of the most highly invasive and highly pathogenic of the group, and grows unusually actively on meat medium. The calf from which this strain was derived was one of several killed by a pellet vaccine. It is conceivable that such an organism as this would be insufficiently attenuated by the conventional procedure, which satisfactorily attenuates organisms of the blackleg group. Careful observation of details of this sort will perhaps clear up most cases of inoculation blackleg.

Animals immunized against the vibrion septique group are not immunized against blackleg, and vice versa. All immune reactions separate the two groups. So far as I am able to discover by a careful search of the literature, there is no sign of failure on the part of one strain of a group to immunize against other strains of the same group.

Robertson (b) found that the four types of vibrion septique which she distinguished on the basis of the agglutination reaction, all produced a toxin which was neutralized by a monovalent antitoxin.

It is my present opinion that cattle should be immunized against both groups, but perhaps such practice should depend on the nature of the organisms found to infest a particular locality. Vaccine for the vibrion septique type should be made separately from blackleg vaccine and probably should be more severely attenuated. It must be emphasized that the worker in charge of the manufacturing of such vaccine should be thoroughly familiar with both types of organisms and should check his cultures continually.

2. THE ANAEROBIC INFECTIONS OF SHEEP

Sheep infections may be considered under four heads: (1) blackleg; (2) malignant edema or vibrion septique infections; (3) braxy or bradsot, and (4) black disease.

(*a*) *Introduction.*—Distinction between these infections is extremely difficult, if we are to judge from the accounts in the literature. One is inclined to make the statement that the nature of the diagnosis depends more on who describes the disease than on what it is. One point to be brought out is that clinical characters have been so emphasized in connection with the different sheep infections that etiologic factors have been largely lost sight of. Another point for consideration is that gangrenous infections of sheep are perhaps different from gangrenous infections of cattle; that is, the specific nature of the host must be considered in diagnosis, as well as the specific nature of the parasite. A possible difference between infections of cattle and infections of sheep appears to be the greater selective preference of the organisms for mucosa of the fourth stomach and the intestines of the sheep. It may well be, however, that closer study will show that this difference is one referable solely to the specific invaders or to the position and nature of the portal of entry of the infection.

Another difference that may exist between sheep and other animals has probably been the cause of much confusion as to sheep infections. This is the phenomenon of the rapid decomposition of sheep cadavers. While cadavers of cattle dead of spontaneous anaerobic infection and those of experimental

animals dead of nonproteolytic anaerobic infection show a marked slowness in decomposition, those of sheep are so frequently referred to as readily decomposing that one must conclude that the nature of the sheep in this regard is different from that of other animals. Krabbe, in 1875, mentions the fact that sheep dead of bradsot decay so rapidly that dogs and ravens will not eat them. On the other hand, we frequentlly hear that the flesh of cattle dead of blackleg may be eaten by man. Jensen, Hamilton, and Gilruth all mention the rapid decomposition of cadavers of sheep dead of braxy. Gilruth (b, 572) goes into some detail in the matter.

(*b*) *Blackleg in Sheep.*—It is evident that many diagnoses have been made of blackleg in sheep when the diagnostician did not know the difference between blackleg infections and malignant edema or vibrion septique infections in sheep. This is, of course, natural, as many diagnosticians do not know the difference between blackleg in cattle and malignant edema or vibrion-septique infections in cattle. Textbooks frequently mention blackleg in sheep, but the actual notes of the occurrence of this disease are rare.

Hadleigh Marsh reports an outbreak of blackleg in sheep. His case is that of a flock of rams into which a ewe was introduced by mistake. The rams fell to fighting and many of them died of a disease which he identified as blackleg.

The invasive organism isolated was typical blackleg (strain RM).

Strain RG, which I isolated from a sheep thought to have died of bradsot on the estate of Graf Bülow in Halle (a sample of the muscle of which animal was sent me by Dr. Jensen), is a blackleg group strain. It is a species distinct from any other in my possession and fulfils all requirements as to blackleg characters as stated on page 388, a.

Various authors report experimental infection of goats with blackleg, but I know of no accurate report of natural infection of goats with this disease.

(*c*) *Malignant Edema and Vibrion Septique Infections of Sheep.*—Records of these infections show that there is a group which must be classified as wound infections.

(*d*) *Braxy or Bradsot.*—The best account of this disease that I have found is that given in the Reports of the Journal of Comparative Pathology and Therapeutics (1902, 15, p. 181). I presume that this review is to be attributed to one of the editors of the journal, Dr. J. M'Fadyean or Dr. Stockman. I quote rather liberally from this paper because some of the sources are inaccessible to me. It is primarily a review of a paper by Professor Hamilton of Aberdeen University, to whom the reviewer refers as the "author." Hamilton

finally identifies the braxy of Scotland with the bradsot of Norway, Denmark, Iceland and Germany. There have been many papers on the disease which lack satisfactory evidence as to its nature (182). "The first really scientific account of the pathology of the disease is that by the Norwegian Government veterinary officer, Ivar Nielsen, who likewise must be regarded as having discovered the micro-organism which is its cause. Previous to the publication of Nielsen's paper in 1888, and even for some time afterwards, the malady was considered to be anthrax."

". . . braxy is certainly not anthrax. . . . There is another disease, however, with which it has even closer points of resemblance, namely, quarter evil (Rauschbrand). Quarter evil is said to be unknown also in Iceland, and the two diseases, and the organisms causing them, when compared, are readily enough distinguishable. Thus braxy is never accompanied by any external lesion, as is the case in quarter evil; it also runs a much more rapid course than that disease.

"Braxy is, therefore, neither an anthracoid disease nor is it to be confounded with any other malady; it is a disease *sui generis*."

(p. 183) "Nielsen has defined it as a gastromycosis, excited by a specific bacillus introduced with the food into the fourth or true stomach, and believes that the disease may either pass into a general affection, or may occasion the death of the animal through absorption of bacterial toxins formed by the organism germinating locally in a part. In these views he was supported by Jensen, who concluded as a result of the researches of previous investigators that bradsot is an acute or even extremely acute, infectious disease which begins as a hemorrhagic inflammation of the mucosa of the fourth stomach, which is accompanied by the formation of gas in the alimentary canal, especially in the stomach, and which kills the animal in some cases by a general infection, in others apparently by toxic poisoning, or possibly in others still by dyspnea caused by tympanites."

(p. 284) "*Age of Animals Attacked.*—Accounts of the disease from all countries seem to uphold the allegation that the first year's animals are far more liable to braxy than those more mature."

Postmortem Appearances.—According to Jensen's description, the following are the chief postmortem appearances:

When the animal is killed during an attack the essential change is a dark bluish-red, somewhat swollen patch on the fourth stomach. This patch increases in size, so that toward the end of the attack a great part or the whole of the stomach may be hemorrhagic or serous hemorrhagic. The fourth stomach and adjacent part of the bowel are devoid of food, but sometimes contain a bloody fluid. The hemorrhagic infiltration may spread from the fourth over the other stomachs, partly over the duodenum, or even over a great portion of the intestine, while other parts of the intestine may be hemorrhagically injected. In the cavities of the body there may be a little serous fluid. The blood is dark, and may be clotted; and the spleen is occasionally somewhat swollen, but may be quite normal. The liver is pale, brittle, and degenerated, and in extreme cases the friability is probably from postmortem causes. The kidneys may be normal or somewhat degenerated; not uncommonly, however, they are enlarged and very brittle or even diffluent. The cadaver decomposes soon, and before long the hind quarters become blown

up with gas, the verge of the anus protuberant, the skin takes on here and there a bluish color, and the wool is easily detached; sometimes the skin bursts and a serous hemorrhagic fluid is seen oozing from the subcutaneous areolar tissue.

The lesions in typical cases are summarized by Hamilton:

"1. The absence of any external manifestation of the disease as in the case of the slough of quarter evil.

2. The tendency which there is, both during life and after death, to the production of gas.

3. The presence of sero-sanguinolent effusions into the various cavities of the body and into the subcutaneous areolar tissue.

4. The tendency to blood-staining of the tissues.

5. The absence of inflammatory manifestations.

6. The occasional, but by no means invariable, hemorrhagic infiltration of the mucous membrane of the fourth stomach, with, from time to time, ulceration or digestion of the surface of the infiltrated parts.

7. The distention with food of the first three stomachs, and the absence of food in the fourth or true stomach, and usually in the intestine. The presence of a little brownish-red grumous liquid in the fourth stomach.

8. The occurrence, occasionally, of hyperemia of the large vessels in the walls of this organ, accompanied by blood-staining of the surrounding tissues.

9. The absence of extensive hemorrhage into the musculature of the body; the absence of gas-production in the muscles.

10. The invariable presence of the braxy bacillus in the liquids, tissues, and organs of the body."

"The organism of braxy is apparently very closely related to that of quarter evil and of malignant edema. The three form a group which stand isolated, and which have intimate mutual relationships. In fact, when growing in solid media there is much difficulty in distinguishing them."

Fröhner described a bradsot-like disease in sheep. He observed the symptoms closely: the animals became depressed, had difficulty in breathing, their abdomens swelled, they were in terrible pain, and they ground their teeth and were restless. They died in a few hours. Decay appeared soon after death. The subcutaneous connective tissue of the head and neck and of the side of the rump showed a gelatinous red infiltration, the muscles of the neck and the back and the cutaneous muscles of the belly were those most affected. The mucous membrane of the nasal cavity and of the turbinates was deep red and swollen, the lymph glands were swollen and infiltrated, the heart muscles grayish red with brownish red spots, the pericardium contained much red watery fluid, the pleura of the ribs were a diffuse deep red and there were also spleen, kidney, and liver lesions. It is possible that these infections took place by way of the nasopharyngeal mucosa, as did the hog cases described by Meyer, as lesions in the head region are not mentioned in necropsies of animals dying of the form of the disease which affects the abomasum, and it is also possible that infection was caused by the bites of flies that could reach only the head of the sheep.

Gilruth (b and c) identifies certain sheep diseases of Victoria and Tasmania and New Zealand with the braxy of Scotland and the bradsot of Scandinavia, and reports an investigation of the diseases of Tasmania and of

Victoria. He makes, however, the mistake of presupposing that the etiologic bacterial agent of a given outbreak is the same in all cases, and therefore he studied carefully only one strain from Tasmania and one strain from Victoria.

Gilruth makes some interesting epidemiologic observations. He connects outbreaks of braxy with dryness and bareness of the soil and with forced ingestion of soil. In common with others he notes that the animals affected are young and in excellent condition. He produces a relative immunity to inoculation by feeding culture to sheep, and holds that this fact disproves, in the present state of our knowledge, the possibility of natural infection by the digestive tract. I cannot agree with this latter statement. Gilruth failed to produce braxy by feeding cultures in a gelatin bolus which contained glass fragments.

Miessner (quoted from Jensen, below) published a paper on the etiology of bradsot in Germany. According to him the lesions produced by bradsot in Germany are not as uniform as in the North, the mucosa of the fourth stomach are less inflamed or not inflamed at all, and bacilli are not always present. His conclusions were that the so-called bradsot bacillus was not the cause of the disease and that the etiology of bradsot was entirely unknown.

Titze and Weichel report extensive experiments with bradsot. Their history of the subject is detailed. They agree with their predecessors that the Scandinavian bradsot and the Scotch braxy and louping-ill closely correspond, but state that no common etiologic factor has been demonstrated for them. (Louping ill was probably wrongly associated with braxy by Hamilton.) They then discuss all reports concerning bradsot in Germany and come to the conclusion that in Germany the disease presents the same characteristics as in the other countries, including the predominance of the disease in winter, but that most of the German cases occur when the sheep are shut up instead of when they are put out to pasture. Moreover, animals of all ages sicken in Germany. The occurrence of the disease is sporadic.

They conclude that the etiology of bradsot is entirely obscure, that the bradsot bacilli are mere cadaver bacilli, and that the diagnosis of bradsot rests on clinical and pathologic characters.

Jensen (c) energetically attacked Miessner, and Titze and Weichel, showing that they may well have considered as bradsot cases that were not bradsot, that Miesner falsely based his accusations that Jensen and Nielsen had worked with old cadavers; also citing protections afforded by vaccination of sheep with bradsot vaccine.

Jensen mentions in this account that of 1,545 sheep that were fed a prophylactic dose of pure culture of the organism, 5 died of typical bradsot; adding that such cases have also been reported from Iceland.

He gives careful descriptions of stomach linings of sheep that died of bradsot in Iceland; these stomachs had been removed immediately after the death of the animal and placed in formaldehyd. Jensen insists that infection takes place in various foci in the mucosa that are hemorrhagic and necrotic; that the bacilli penetrate at these spots and enter the loose connective tissue of the submucosa, there multiply extensively, and then invade the whole mucosa. His illustrations seem adequately to substantiate his statements.

I fully agree with Nielsen in his interpretation of the effect of cold on these infections. We know from many records of human pathology during the war (Taylor, Hartley) that any influence tending to decrease the circulation in a part allows in that part the development, even in the absence of necrotized tissue, of a gas gangrene which would never have occurred had

circulation been plentiful. The blood, with its leukocytes, is in these diseases the greatest bodily defense. It is most likely, indeed, that chilling of an animal will permit a multiplication of toxic invaders which would never have occurred under normal circumstances. The factor may be looked on simply as a dilution in health, or a concentration, in the case of poor circulation, of the toxin surrounding the first invaders.

Jensen and Nielsen believe bradsot to have its portal of entry primarily in the fourth stomach. Hamilton believes they overemphasize this conception. Nielsen's statement as to the shearing of sheep in the fall in Norway is suggestive of wound infection. It is, however, more than probable that alimentary canal infections may take place. Many infections are mentioned in which no external wound is to be found. I think that the possible minuteness of the necessary portal of entry for these organisms, whether it be external or in the alimentary canal, has been overlooked (see p. 423).

(*e*) *Black Disease*.—In New South Wales a disease of sheep exists which has been thought by some to be an anaerobic invasion. Dodd reports experimental work on this "black disease."

Black disease is very erratic in making its appearance; it is enzootic, occurring in the southern highlands of New South Wales. It appears in late summer (February) and ceases in early winter (May), usually, but not always vanishing with the disappearance of frost. Inland it is a winter disease; the winters inland are warmer than they are farther south. Animals of all ages and of both sexes are affected; the disease is apparently peculiar to sheep; the mortality is variable and sometimes heavy. Stricken animals show few symptoms, they lag behind the others, and die quickly. Necropsies of animals dying of natural infection show thin bloody liquid in the abdominal and pleural cavities and some congestion of the abomasum, occasional injection of the intestine, and injection of the skin and subcutaneous connective tissue. Decomposition appears early. Heart blood of animals found recently dead of the disease, when inoculated into normal sheep, produces local gelatinous edema, thin blood-tinged serous infiltration of the subcutaneous connective tissue, and occasionally gas. Some muscles show a dark hemorrhagic appearance not unlike that found in blackleg cases, and they have a distinct peculiar but not putrefactive odor. Other affected muscles have a clay colored appearance and on incision have a distinctly putrefactive odor. The abomasum is usually congested, and the intestines are occasionally injected, sometimes markedly so. Feeding experiments were negative. Tissue inoculations and culture inoculations sometimes killed and sometimes failed to kill guinea-pigs and rabbits, being more highly pathogenic for the former.

The work mentioned above was done in 1914 with material from animals found dead of black disease and thought to have died only a comparatively short time before necropsy examinations were made. Later research showed that it was difficult to produce the disease in animals with material and cultures from sheep that had just died of black disease. Dodd says: "The conclusion to be drawn from the research work narrated is that the bacteria isolated from the blood, exudates, organs, etc., of sheep found dead of black disease, including that of the braxy type, are agonal or postmortem invaders."

To me it is quite conceivable that black disease may be an alimentary canal invasion by a highly toxic vibrion septique type of organism that kills the

sheep by its toxin and becomes septicemic only shortly before death, escaping in such small numbers that inoculation of fresh tissue not directly involved in the primary process will not reproduce the disease in some cases, whereas the inoculation of tissue that has lain for a few hours will do so in all or nearly all cases. Nielsen (Jensen, b) found that the bradsot bacilli do not always become septicemic in sheep.

We have record of cattle infections (Wulff and Laabs, see reprint) that were diagnosed as B. chauvoei invasions, but some of which may have been invasions of the vibrion septique group, which show no muscle lesion at all. We have record of hog invasions by strains of the vibrion septique group, in which no external lesion was to be discovered and muscle lesions were absent, and in which the resemblance to braxy was marked. It is to be expected that material from such cases when inoculated subcutaneously or intramuscularly into the thigh of an animal will produce edema and sometimes gas at the site of inoculation. But Dodd's organisms and Gilruth's organisms and also Titze and Weichel's organisms reproduce, on inoculation, also the injection of the abomasum and of the intestine and other lesions produced by the disease in its natural form. It is to be expected that in the case of artificial subcutaneous or intramuscular inoculation death will occur before the mass infection in the alimentary canal is as great as in the case of internal infections. The injection of the membranes will then also be less than in the case of an alimentary canal invasion. Vibrion septique strains produce strong toxins, and when an infection is under way, it is a matter of only a few hours before death ensues.

Dodd is right in holding a skeptical attitude in studying a disease of this sort; he is to be commended for choosing fresh cadavers for his investigations. He is quite right in saying that the finding of a highly pathogenic anaerobe in the tissues of a dead sheep is no proof that that anaerobe was the cause of the death of the sheep. But the finding of a highly pathogenic anaerobe in the tissues of an animal not long dead demands careful investigation as to its location and quantitative distribution in the body of that animal before one is warranted in stating that the anaerobe was not the cause of the death of the animal. I feel that Dodd entirely underrates the pathogenicity of the organisms of the vibrion septique group, and confounds them with "cadaver bacilli." This is easily understood because of the prevailing confusion on this point. It must be emphasized that the highly pathogenic anaerobes are members of definite restricted groups that are few in number, and that there are a vast number of species of anaerobes that may be called "cadaver bacilli" and have no invasive power of their own. Lumping all anaerobes together as "cadaver bacilli" because most of them are putrefactive is as primitive a procedure as would be the lumping together of all aerobes for a similar reason.

In questioning the anaerobic origin of black disease Dodd is also forced to question the anaerobic etiology of Gilruth's nonwound infection braxy cases. This he has a right to do as Gilruth's observations are not founded on sufficient data to be conclusive. He also questions the anaerobic etiology of the northern braxy. I feel that it would be necessary for him also to question, on the same ground, all anaerobic infections of cattle in which no external lesion is to be found—which, according to Wulff, is true of the vast majority of blackleg cases. The parallelism between the anaerobic cattle and hog infections and these sheep diseases is close.

Grosso identifies the bacillus of bradsot with the malignant-edema bacillus by means of the agglutination reaction.

Zeissler (a) states that the bacillus of Ghon and Sachs is the cause of bradsot in sheep, goats, and hogs, including wild hogs. He and Eugene Fraenkel found the bacillus of Gohn and Sachs in bradsot in a sheep and a goat. I presume that the statement regarding hogs is based on the work of Köves (see reprint). Zeissler asserts that the bacillus of Gohn and Sachs is the one specific cause of bradsot.

(*f*) *The Epidemiology of the Anaerobic Infection of Sheep and Tentative Conclusions as to Such Infections.*—We know that anaerobic invasion of sheep by organisms of the vibrion septique group and of the blackleg group may take place after wound contamination. This phenomenon is entirely analogous to that of cattle infection.

Other infections of sheep, which do not follow the contamination of visible wounds, do not present a patent parallel to cattle infections. The striking differences are two: First, anaerobic infections of sheep are usually caused by organisms of the vibrion septique group and rarely caused by organisms of the blackleg group, and anaerobic infections of cattle are usually caused by organisms of the blackleg group and less frequently by organisms of the vibrion septique group. Second, the infections of sheep are more often localized in the alimentary canal and serous lining of the peritoneal cavity, while in cattle the infective process is more frequently localized in the muscles. Granted that the predominating invaders of cattle, namely, the organisms of the blackleg group, prefer muscle tissue, and the predominating invaders of sheep, the organisms of the vibrion septique group, prefer somewhat the serous membranes, I do not believe that this fact alone explains the great number of alimentary canal localizations in sheep. There must either be a constitutional difference between sheep and cattle in this respect, or there must be a difference in the mode of infection in the two species. It seems to me that a satisfactory explanation of the differences would be covered by two considerations: first, the acceptance of Nielsen's and Jensen's contention that alimentary canal infection is common in sheep; second, the consideration that if insects may transmit these diseases, cattle would be far more exposed to infection of skeletal muscle than sheep, for sheep may be bitten by free flying insects over a very small portion of the body. The fact that bradsot is common in winter and blackleg is common in summer fits in closest harmony with these considerations. Moreover, on inoculation, blackleg is highly pathogenic for sheep, and vibrion septique strains are highly pathogenic for cattle, and a difference in the susceptibility of the two types of organisms for these two types of transmission may explain the discrepancy in the predominance in natural infection of one group in cattle and of the other in sheep.

Data concerning sheep infections are not sufficient to permit a discussion of insect transmission. We may, however, consider more closely the question of alimentary canal invasion. Jensen's evidences as to the mode of invasion in the bradsot of Iceland are probably sufficient, though the reason for such a mode of invasion is unknown. In Germany and in other countries, however, where the lesions are not so uniform, other factors must be considered. The fact that intramuscular inoculation of vibrion septique strains into guinea-pigs and sheep produces gastro-enteritis makes necessary the careful search for a minute portal of entry in bradsot cadavers. This has been pointed out by Hamilton and by Gilruth. The suggestion that organisms of the blackleg group do not show the preference for growth on the abdominal serous membranes that is shown by strains of the vibrion septique group would be worth

considering in the case of suspected bradsot with slight alimentary canal lesions. Vibrion septique strains also vary somewhat among themselves in respect to production of gastro-enteritis in the guinea-pig.

I hope that after the publication of this article it will no more be stated that because bradsot material was capable on experimental inoculation of producing "malignant edema," bradsot cannot be considered to be caused by an anaerobe. No one who has studied the action of anaerobes in the animal body would for a moment hesitate to declare the possibility that bradsot may be an alimentary canal localization of the disease known as "malignant edema." These diseases are analogous to the group caused by one specific organism: B. anthracis. The diseases caused by this organism are known as malignant pustule, woolsorter's disease, and intestinal anthrax—depending on the site of the multiplication of the organism, and on the mode of transmission and inoculation. Infections of the subcutis and muscles by organisms of the vibrion septique group are called "malignant edema"; infections of the fourth stomach and intestine of sheep by organisms of the vibrion septique group are known as "braxy" or "bradsot"; vibrion septique infections of the lungs and pleural cavity are termed "pneumonia." It is time that more emphasis were placed on the specific etiology of diseases and less on the lesions produced by them.

The chaotic condition of the subject of the anaerobic infections of sheep and the diseases that resemble them is, to my mind, due to several causes. First, although the disease of braxy has long been considered an infection, many of the investigations concerning it have been undertaken from the pathologic-anatomic point of view. Second, when infections have been considered probable, the idea of a specific causative organism has been held to and few cases have been properly studied. Third, the epidemiologic rules applied to infectious diseases in general have been applied also to these diseases, which differ from ordinary infections in that they are caused by accidental invasion by organisms that are frequently present in the alimentary canal of the host: organisms to which the host usually shows immunity. Therefore mechanical, physical, and chemical factors must be considered as inducing alimentary canal invasions by anaerobes, and the closest analogues to such invasions are to be found in wound infections and not in contagious infections. Common sense and not dogma should be our guide in seeking the etiologic elements concerned in these diseases, which certainly cannot be included in those infections that must be judged by the four rules of Koch.[11] Feeding experiments have amply proved that these diseases are not transmissible diseases in the ordinary sense. Certain factors to be considered in anaerobic alimentary canal infection are:

1. Gross wound infection in a normal animal. The mucosa being cut by a foreign object or deeply pricked by a sharp point, may become contaminated by a toxin producing organism, and wound infection is far more likely to occur

[11] Koch himself says as to the epidemiologic considerations involved in wound infections (a, 75): ". . . ein vollgültiger Beweis . . . nur dann geschafft werden kann, wenn es gelinkt, die parasitischen Mikroorganismen in allen Fällen der betreffenden Krankheit aufzufinden, sie ferner in solcher Menge und Verteilung nachzuweisen, dass alle Krankheitserscheinungen dadurch ihre Erklärung finden, und schliesslich für jede einzelne Wundinfektionskrankheit einen morphologisch wohl characterisierten Mikroorganismus als Parasiten festzustellen.

"Sollte es denn nun aber möglich sein, diese Bedingungen überhaupt jemals zu erfüllen?"

(100) "Denn je länger ich mich dem Studium der Infektionskrankheiten befasst habe, um so mehr, habe ich die Ueberzeugung gewonnen, dass das Generalisieren neuer Tatsachen hier verfrüht ist und dass jede einzelne Infektionskrankheit oder Gruppe nahe verwandter Infektionskrankheiten für sich erforscht werden muss."

than in the case of an infected external wound of the same size for two reasons: the conditions are more anaerobic, and the infective agent probably is usually present in the vegetative form.

2. The infection of a minute wound in a debilitated animal—such a case as that suggested by Nielsen in which freshly shorn sheep are turned out in the cold. Also animals sick from some other cause may come under this head: Kitt's cases of hogs dying of Schweinepest and anaerobic invasion (see reprint) would probably come under this head. Perhaps also Meyer's cases (b) of cattle with liver foci, which were apparently caused by organisms of the vibrion septique group that were nonpathogenic to cattle on artificial inoculation. I believe that abundant cases of this kind might be collated from human necropsies.

3. Infection of wounds either in the alimentary canal or in the liver; these wounds are caused by flukes or other parasites (worms are common in sheep). This possibility has been emphasized by Gilruth.

4. It is quite conceivable that organisms of the toxic type may sometimes find suitable food material so abundant in the alimentary canal that they multiply to such an extent that their toxin overcomes the general resistance of the wall of the alimentary canal and they invade en masse. The "bacterial felt" pictured by Jensen (plate 3) is suggestive of such a condition, and Jensen has always held this form of invasion to be the usual one. Debilitated animals would of course be most likely to succumb to such toxic assault. One may well conceive, however, that in a healthy animal in which some intestinal stasis occurred, or in one in which some highly concentrated suitable food were present for the rapidly multiplying anaerobes to grow on, an unusual multiplication of organisms might occur, and invasion might, in isolated cases, take place.

5. Some chemical agent in food (for example, poisonous plants) may break down the resistance that the animal tissues offer to the toxins and aggressins of the organisms.

6. The ingestion of earth may, as suggested by Gilruth, promote anaerobic alimentary canal invasion.

A final criticism of the research performed on this subject is in place. To my knowledge, a careful, thorough investigation either of the mixed flora or of pure strains of the anaerobes involved in the alimentary invasions of sheep has never been made. Elaborate technic for such study has not existed, but even such technic as has existed has not been applied. The study of the anaerobes is difficult and time consuming and the anaerobic field is enormous. The veterinary pathologist cannot alone hope to solve the problems involved, unless he become bacteriologist as well and spend many long months in the study of the organisms with which he experiments. Unless the investigator has time for such study, experimental research on this complicated problem will lead nowhere. It is a problem to be undertaken seriously by a laboratory having ample funds and many experimental animals and by a worker who is able to devote his full time

to the subject. If he have the services of a trained epidemiologist, so much the better. Fragmentary inoculation experiments with organisms of whose nature and relative distribution we know nothing lead us to confusion instead of to knowledge.

3. REINDEERPEST

A discussion of this disease must not be omitted.

Lundgren first reported it in detail in 1898. He brought with him from Lapland several tubes of pericardial and peritoneal and pleural fluid from a reindeer on which he made a necropsy examination. This material served for an elaborate and painstaking investigation by Bergman, who published an account of his work in 1901.

So far as these two publications show, there has been reported by an experienced pathologist only one necropsy examination on a reindeer supposedly dead of the spontaneous form of the disease, namely, Lundgren's case. Bacteriologic investigation is reported on material from this single case. Identification of this case with the other cases of the diseases known as reindeerpest rests entirely with the Lapps. The Lapps are a somewhat primitive people of exceedingly keen observation and scientific perception; especially, as Bergman says, may they be depended on to report with accuracy observations concerning reindeer. But I do not think that Lundgren and Bergman are justified in concluding that the pathogenic organism which was evidently the cause of the death of this reindeer is the one etiologic agent involved in the causation of reindeerpest or even in calling it the bacillus of reindeerpest.

Reindeerpest is a disease of the summer time and of the valleys. Reindeer are kept for most of the summer in the mountains on account of insects, and if they are brought down to the valleys they may die in large numbers of reindeerpest. When the herd is taken again to the uplands the disease disappears, when brought down again it reappears, only to die out in September. Epidemiologically the disease differs markedly from the diseases already discussed in only one respect, namely, that a large number of animals are attacked in a herd. There are three possibilities in its epidemiologic classification: that it is a contagious disease, or as Lundgren suggests, that it is an insect-borne disease, or that it is a disease introduced through abrasions or wounds, which diseases are probably governed in their ability to infect by several factors that may vary in different localities. Lundgren notes, for example, that during the summer when reindeerpest was most prevalent there was also a great deal of hoof disease among the animals, and he suggests that the pest originates as an infection of the diseased hoofs that partakes of the nature of a wound infection. The conditions in Lapland are different in many ways from those farther south. If it is true that the incidence of the disease ceases when the animals are driven into the mountains, the contagious nature of the disease is thrown into doubt, thus making it less likely that it is of the hemorrhagic septicemia type. Insects are numerous during the summer in the valleys of Lapland, and the reindeer is one of the animals most persecuted by them. The incidence of the disease in valleys and its occurrence in summer resembles the incidence and occurrence of blackleg. Valleys are highroads and are muddy; insects are there most abundant. The Lapps are an inquiring people. Bergman says that they often perform a necropsy examination on their dead reindeer to find out the cause of death, and they keep and use the skins and

let the bodies lie. The latter conditions favor anaerobic infection. As to the natural mode of infection of this disease, nothing is known. Young animals are the victims, even very young ones.

Bacterial Studies: Four of the five samples brought to Bergman by Lundgren were contaminated by mold. Bergman reproduced with them in various animals a disease in most ways similar to the disease in the reindeer on which Lundgren performed necropsy examination. From the organs of these animals and from the transudates of the original animal Bergman invariably grew an aerobe that was a facultative anaerobe. This aerobe grew in colonies on the surface of gelatin plates. Bergman describes it minutely. In culture it grew abundantly in the form of heavy rods 1.6x0.8 mikron, and reluctantly formed spores. It was a gas and acid former and grew on all mediums. It resembled several strains of sporulating aerobes that I have encountered. In the animal body the morphology of the organism, as shown by Lundgren's photographs and by Bergman's photographs, is most astoundingly like that of an organism of the vibrion septique group. Moreover, Bergman's bacillus forms long chains on the peritoneal surface and liver surface of animals. His account of the mode of spore formation of his bacillus is also characteristic of that of a vibrion septique type of organism.

Bergman's bacillus was highly pathogenic for reindeer, sheep, guinea-pigs and white mice, and it was also pathogenic for a calf, cats, brown rats, pigeons, and sparrows, and for frogs at 27 degrees. It was not pathogenic for rabbits, hogs, dogs, and chickens. I would suggest that there are not many species of organisms known that are so widely pathogenic. Bergman immunized sheep with sublethal doses of his organism. This animal was not immune to blackleg.

The pathogenicity of Bergman's material decreased rapidly in liquid cultures and not quite so rapidly in cultures on solid mediums. Pathogenicity could be recovered by animal inoculation.

We have here before us two possibilities: First, An aerobic organism exists (and we know of no other such aerobes) that is most strikingly similar (in morphology, incidence, pathogenicity, and in the lesions that it produces) to the organisms of the vibrion septique group, but which rapidly loses pathogenicity in aerobic cultures. Second, Bergman had to do with a symbiotic mixed infection of an organism of the vibrion septique group and an aerobic sporebearer, the latter able to render conditions suitable for the growth of the former on a plate. One hesitates to suggest the second contingency, for Bergman was evidently a careful worker and himself tried to demonstrate the absence of such a possibility; but it seems to me that such a possibility cannot be ignored. Bergman does not tell us of a careful examination of anaerobic colonies, or whether anaerobic cultures lost their virulence rapidly. He tells us of a dried gelatin plate with colonies, individual ones of which were nonpathogenic. "But if there were in the whole plate only one colony that contained

virulent reindeerpest bacilli, that would be sufficient to infect a reindeer." So he washed off the plate and inoculated the suspension into a reindeer and produced the disease.

I should suggest that if the losses from reindeerpest are considerable today, it would be worth while to study many cases of the disease, or at least several cases from various valleys. Investigation of the diseases of reindeer is, however, according to Lundgren, an extremely arduous pastime. Bergman's work is interesting and a step in the right path, but little can be done with material from one specimen. It must be borne in mind that anaerobic invasion may at times be a fatal one secondary to another type of infection.

4. THE ANAEROBIC INFECTIONS OF THE HORSE

I believe that earlier reports tend to diagnose horse infections as blackleg more frequently than do later ones.

Arloing, Cornevin, and Thomas (86) failed repeatedly to produce more than a local swelling in horses and asses by inoculating blackleg virus.

Hutyra and Marek (p. 34), in referring to malignant edema, say: "For natural infection of domestic animals solipeds are most susceptible."

Diedrichs reports investigation of material from the two horses that died of a Rauschbrand-like disease.

The material from the first horse produced what were apparently mixed infections fatal to guinea-pigs and pigeons. Cultures failed later to kill guinea-pigs. The culture was identified as Pseudorauschbrand in the Hygienic Institute of Berlin. The second strain remained proteolytic through several inoculations; but a guinea-pig, inoculated with a culture that had had several animal passages had little putrefactive odor and showed a colorless edema as thick as a finger; no gas is mentioned. The mixed (proteolytic) culture killed rabbits and was somewhat pathogenic for pigeons. It would seem that oedematiens-group infection is not improbable in this case. Infections of the vibrion septique group are probably never accompanied by proteolytic organisms through several passages. They do not produce edema as thick as a finger, and they produce gas. According to Diedrichs, the organism was not blackleg, and the pathogenicity tests apparently bear him out.

Von Hibler reports no cases of anaerobic infection in horses. He isolated B. oedematis maligni "in sinne von Koch" (Von Hibler X) from a mule.

Weinberg and Séguin report (b, p. 320) the case of a mule used for making antivenin. As a result of the venin inoculation a mixed infection of the head and neck developed. Edema was extensive, as was the tissue destruction, the latter being almost unbelievable in its extent. The infection was apparently due to B. histolyticus and B. oedematiens. The animal recovered after inoculations of mixed serum.

No samples of material from horses had come to this laboratory until Dr. Wood was so kind as to furnish me with four specimens, which were derived from horses that were being used for an immunity experiment. The pathogenic strain from one of the four horses was a typical vibrion septique: of interest is the fact that the animal, which had developed an extensive edema, recovered spontaneously from the infection.

The three other samples contained an interesting organism. Careful tests showed the three strains to be alike. The infected horses all succumbed, showing on section edema and gas formation. This organism (strains O 55, O 51, O 09) behaves much as do oedematiens strains. Inoculation of 0.5 c c of 24-hour meat medium culture of this bacillus into the thigh of a guinea-pig may kill the animal in 18 hours or less without any invasion of the body by the bacilli, or if little toxin be present the infection kills in 24 hours with septicemic invasion. A white gelatinous edema, that does not rapidly lose its gelatinous consistency on section, is found to surround the inoculated region. It is slightly blood stained. No gas formation takes place. A marked injection of the abdominal serous linings is the notable internal lesion.

On meat medium the organism produces a little gas, occasionally turning the meat pinkish, but producing so little acid that the pink color soon fades. The short, rather heavy bacilli are scarce in the medium, they stain palely as a rule, do not sporulate actively, and in 48 hours many individuals show the peculiar ghost-like appearance characteristic of oedematiens strains. Auto-agglutination takes place. The growth on milk is like that of B. oedematiens: reluctant and slow, very little acid being produced. On blood broth H_2S is produced, otherwise proteolysis is not patent. In colony formation, however, this organism is definitely to be distinguished from all the oedematiens-group strains in my collection.

Christiansen (a) found that normal horse serum protects guinea-pigs against infection with B. welchii.

I think that it can be stated that horses are subject to infection by organisms of the vibrion septique group and that they are subject to infection by organisms of the oedematiens group. I do not believe that any case of true blackleg infection in the horse has been established.

5. ANAEROBIC INFECTIONS OF THE HOG

It is apparent in the case of hog infections, as it was in the case of horse infections, that early diagnoses were more generally referred to blackleg and more recent diagnoses have been referred to the organisms of the vibrion septique group.

Arloing, Cornevin, and Thomas (p. 89) failed on repeated trials on all types of hogs to produce blackleg by inoculation. They state (p. 90) that hogs are susceptible to gas gangrene.

K. F. Meyer (a) reviews this subject. "The organism isolated from hogs by Marek, even though morphologically identical with B. chauvoei, had a high pathogenicity for rabbits; the publications of Marek do not contain any detailed account as to the biochemical actions of the observed organism. The same remarks apply to the publications of Born and Battistini in which, based purely on morphologic similarity of the observed organisms in the diseased muscles, the diagnosis of symptomatic anthrax in hogs was made." Meyer reviews the transmission experiments of von Ratz and holds that the lesions described by von Ratz so closely resembled those produced in hogs by the bacillus of Ghon and Sachs as identified by himself that he doubts, in the absence of cultural studies, whether von Ratz was really working with true symptomatic anthrax. He calls attention to the work of Glässner and of Wulff, who attempted to infect hogs with blackleg material and failed to produce more than a local swelling. Meyer says: "I cannot therefore agree with v. Ratz that hogs are susceptible to symptomatic anthrax."

Dr. Haslam has sent me a culture from one of several hogs that died of an anaerobic infection. The organism is a typical strain of the vibrion septique group.

Von Hibler reports a case of infection in a wild hog that was caused by B. novyi.

The lesions resembled those of Rauschbrand. Von Hibler was in possession of Novy's original strain of B. oedematis maligni II. This strain and others resembling it do not in my hands produce in the guinea-pig an infection remotely resembling blackleg. B. oedematiens Weinberg, strain Joly, does, however, produce a black hemorrhagic edema, as black as that produced by blackleg organisms. B. oedematiens and B. oedematis maligni II. are closely related and should logically be placed in the same group. It would be interesting to examine the strain isolated from the hog by von Hibler. One must remember that a hog and a guinea-pig might show different lesions in the same infection.

Von Hibler also isolated B. welchii (B. phlegmones emphysematoseae) from a hog that was thought to have died of pest.

I think it may be stated that hogs are susceptible to infection by organisms of the vibrion septique group, especially when they are weakened by other diseases, or when they are wounded. I do not believe that there is only one such invader that can be termed specific.

The question of invasion of hog tissue by organisms of the blackleg group is to my mind still an open one. These organisms are in general less pathogenic than those of the vibrion septique group. To prove anything in regard to this point a number of indubitable strains of the blackleg group of differing characters should be used. Probably exhaustive study would show that strains of the blackleg group are distinctly less pathogenic for hogs than strains of the vibrion septique group, and are incapable of infecting sound tissue of healthy animals, but that, were a wound severe enough, a blackleg strain would be capable of penetrating hog tissue, as was the Welch strain in von Hibler's case. The organisms of such wound infections should be looked on as relative in their pathogenicity; their action depends on so many factors that they cannot be considered in the light of our conceptions of ordinary infectious diseases.

Oedematiens group organisms are pathogenic for hogs (von Hibler).

B. welchii (B. phlegmones emphysematoseae, Fränkel) may be pathogenic for hogs. Von Hibler does not state how his hog may have contracted the infection.

6. ANAEROBIC INFECTIONS OF THE DOG

Evidently dogs and cats are more highly resistant to anaerobe infection than are herbivores, or we should hear more of such infections. It must also be borne in mind that carnivores are far more agile and skilful animals, and in the domesticated state are probably far less subject to wounding than are the herbivores.

7. AN EXPERIMENTAL INFECTION IN A CAMEL

An experimental infection in a camel is reported by Cross. He inoculated with blackleg virus a 7 year old camel and 2 yearling camels. All died of the infection.

8. ANAEROBIC INFECTIONS OF GAME

Ott and Ströse state that malignant edema is rare in game animals and that Rauschbrand has not been reported in game animals.

Budd, in 1863, quotes Hintermeyer who described in 1846 an outbreak of "quarter evil" in a herd of deer in a park in Germnay. There was an exudate from the nose, anus, and vagina of the animals. Just what the disease was we can probably not determine.

9. ANAEROBIC INFECTIONS OF RODENTS

Guinea-Pigs: I have observed two cases of spontaneous infection in the guinea-pig, which were caused by organisms of the vibrion septique group.

I believe that, in general, mice, rats, and rabbits are less susceptible to anaerobic invasion than are guinea-pigs, but this may be merely an impression. Cornevin found the guinea-pig most susceptible to experimental anaerobic infections.

10. WHALE SEPTICEMIA

In 1888 Nielsen (b) discovered and described a septicemia of whales that was due to wound infection. The natives of a region near Bergen caught whales by shooting them with infected arrows and then waited a day or two for the infection to develop before harpooning the animals. Nielsen described the causative organism of this infection and declared it to be similar to (but not identical with) the causative organism of blackleg. Recently M. Christiansen (b) has made a close study of an organism isolated by him from several samples of muscle from a single whale, which were taken in the late eighties. He places the organism in the same group as the bacillus of Ghon and Sachs, for which he proposes the name "Ghon-Sachs group," and he shows various characters differentiating his organism from B. chauvoei. Morphologically and pathogenically Christiansen's organism is distinctly to be placed in such a group, in spite of the fact that he insists that it is not flagellate, which character he believes to be an unimportant one. I have myself isolated an organism from whale muscle, which was kindly furnished me by Dr. C. O. Jensen. Dr. Christiansen states that my strain is identical with his strains; they all come from the same whale. I find that my strain is definitely referable to the vibrion septique group, but it is markedly distinct from any other strain in my possession. I should be much surprised if only one species were involved in all these wound infections of the whale. Very likely, however, one species was more pathogenic for whales than others and so became the predominant or more usual invader.

Obst found in 287 swelled cans of sardines an anaerobic organism "in pure culture." Assisted by W. G. Smillie she identified the organism as B. wallfischrauschbrand Nielsen. No animal experiments are recorded, and no standard anaerobe mediums were used in this study. The organism formed gas from protein and carbohydrate mediums; a foul odor was present in broth cultures. It sporulated heavily, forming "round" spores, giving the bacillus a "tennis racket formation." I can see no possible justification in thus identifying an anaerobe. It is counter to all chances that the anaerobic organisms in the 287 cans were in pure culture in the cans, and that the organisms in the 287 cans were the same. There is probably ample justification for identifying the bacillus of Christiansen with that of Nielsen. This organism is not proteolytic and forms oval spores. The aerobist should be warned that there are a thousand chances to one that a stray proteolytic anaerobe picked up from anywhere has not been described. Moreover, the possibility of any accurate identification of such an organism from a printed description is slight. The most we are today justified in doing when we wish to identify an anaerobe is to determine its group affinities. A careful and detailed description of a pure culture of the organism is then in order. Anaerobic literature is in no condition to be referred to for specific identifications of more than a scant handful of organisms; an authoritative systematic work on the subject does not exist.

11. NONMAMMALIAN ANAEROBIC INFECTIONS

Birds seem to vary in their susceptibility to anaerobic infections. Fowls are usually or invariably reported as immune. Pigeons are apparently subject to artificial infection with some strains of the organisms of the vibrion septique group but not to blackleg; they are highly susceptible to Welch bacillus infection. Sparrows may be killed by some vibrion septique strains.

Arloing, Cornevin, and Thomas (p. 91) produced infection by blackleg in frogs that had been kept at 22 C. in water. The organism of Bergman (see p. 438), which is not proved to be an anaerobe, infected frogs kept at 27 C. in air.

12. THE NOMENCLATURE OF ANAEROBIC DISEASES

It has probably been noted that the subject of the terminology of anaerobic infections is in an exceedingly chaotic condition. This is primarily because human and veterinary clinicians and pathologists have frequently attempted to diagnose the diseases seen by them according to the causative organism that they imagine is involved in a given case, instead of describing the disease as they find it and sending properly collected specimens to the bacteriologist for examination. Moreover, it is my experience that an opinion based on clinical and microscopic findings early in the examination of material from a case, is likely to prove mistaken or incomplete. Far too often has the bacteriologist, who found in a gas gangrene sample the ubiquitous B. welchii, contented himself with this finding and searched no farther.

Ghon and Sachs, who carefully reviewed the subject of nomenclature in 1903-04, came to a very simple conclusion, which, were the facts as they then appeared to me, could be accepted as a basis of terminology. Their idea was to term "malignant edema" the disease caused by the Bacillus oedematis maligni (under which name they identified Koch's, Pasteur's, and their own organism) and to call "malignant emphysema" the disease caused by Bacillus phlegmones emphysematoseae, Fraenkel. They state that this system has its shortcomings, but they do not wish to introduce more terms into the nomenclature.

Not to introduce new names when new conceptions arise would be hyperconservative; but it would seem also that the definition and application of old names needs revision. The whole field of anaerobic invasion must first be glanced over. We have a number of diseases to consider, which vary:

1. According to their location in the animal body: subcutis, muscle, pleural and peritoneal linings, glandular organs, alimentary canal.

2. According to their mode of inoculation: wound infections demonstrable as such, and infections whose point of entrance is obscure.

3. According to their distribution from the site of inoculation: through lymph or blood channels, through muscle or connective tissue spaces, or along lining surfaces.

4. According to the specific nature of the host, its age, and its condition.

5. According to the primary group affinities of the invaders: proteolytic, nonproteolytic, or mixed proteolytic-nonproteolytic.

If the infection is nonproteolytic, it varies:

6. According to the species of the invaders involved: (I have good reason for believing that wound infections, at least, are far more frequently and more highly polyspecific than they are usually thought to be), or according to the species of the one invader.

7. According to the relationship between the toxicity, the aggressive powers, and the reproductive powers of the particular strain or species of the principal invader, on which depend edema production, gas production, transudate formation, muscle invasion and hemolysis.

The varieties of specific invaders may be thought of as moderately numerous.

It is, according to the above desiderata, quite ridiculous longer to chain any specific invader to any pathologic term. Such an effort would be futile and would continually embarrass the pathologist. What we need is a set of purely pathologic diagnostic terms; descriptive terms we have in plenty. We may use old names for the pathologic processes encountered whenever they are descriptive of the lesion to which they are applied; but in so doing we must discard entirely the implication of the expression of an opinion as to the identity of the organism causing the lesion. The human and veterinary pathologists sorely need such a system as this whenever the material that passes their hands is to be examined by a bacteriologist. The pathologist cannot possibly be expected to determine the specific cause of an anaerobic invasion. He must have not only the descriptive terms that he possesses, but also diagnostic terms that do not in any way commit him to specific or even generic bacteriologic determination. The identity of such terms is for the pathologists to decide on. The bacteriologist is then free to make a diagnosis of the group or specific affinities of the invading organisms. I propose, however, that the terms malignant edema be given to the pathologists to abandon or to use as they see fit, and the term "bacillus of malignant edema" be discarded forever from bacteriologic terminology. The name B. oedematis maligni will also have to be abandoned unless someone, somewhere, can produce a culture whose history can be traced to Koch's laboratory, and which reproduces, on subcutaneous inoculation into laboratory animals, the lesions described in "Zur Aetiologie des Milzbrandes." No literary identifications of other strains with that of Koch's description can scientifically be accepted. Adamson's proposal to use the name B. oedematis maligni for the sporogenes type of organism has no systematic precedent, is illogical, and is likely to increase the existing confusion in our terminology.

The use of the term "gas bacillus" is likewise to be discouraged because it has become more or less definitely associated with B. welchii, which is no more a gas bacillus than are the vibrion septique organisms.

The anatomic diagnosis of "Rauschbrand" or "blackleg" should, in my opinion, for legal purposes, include infections by members of the blackleg group and of the vibrion septique group, or else some other term should be devised to include both types. There is no logic in separating the two diseases and recompensing the farmer for cattle dying of one and not of the other; moreover, different local veterinarians will probably never agree in their diagnoses of these diseases. The bacteriologist's diagnosis should depend on the organism or organisms found, and the two workers, pathologist and bacteriologist, can thus work in harmony.

I do not think that we are as yet in a position to take a stand as to the terminology of the infections of sheep. There should be a term (and bradsot and braxy probably fill the bill) that is descriptive of a rapidly fatal disease of the alimentary canal, which is associated with abundant multiplication of anaerobic organisms in the tissue. No specific or even generic meaning should be implied by the expression "anaerobic organisms." "Bradsot" or "braxy" may be taken to apply to such a disease in sheep or in goats or in hogs, as Zeissler has used it, or in other animals as well (see cattle, p. ??). When a thorough investigation of the obscure cases in sheep has been made it will be time to revise the nomenclature of such diseases.

GENERAL CONCLUSIONS

For more detailed conclusions see pp. 388, 389, 411, 416, 419, 420, 425 and 434.

Cattle are subject to spontaneous infection by organisms of the blackleg group and, somewhat less frequently, by organisms of the vibrion septique group, both of which types of infection are usually diagnosed as "blackleg."

Sheep are subject to spontaneous infection by organisms of the vibrion septique group and somewhat less frequently by organisms of the blackleg group. Both types of infection are probably diagnosed at times as braxy, as blackleg, and as malignant edema. It is possible that other diseases that are not of anaerobic origin are at times diagnosed as "bradsot" or "braxy."

The possibility exists that reindeerpest, as described by Lundgren and Bergman, is an anaerobic infection.

Horses are subject to infection by members of the vibrion septique group. Such infection may or may not follow a wound. It was formerly frequently diagnosed as blackleg. Horses are also subject to infection by organisms of the edematiens group. True blackleg group infection in the horse has probably never been demonstrated.

Hogs are subject to infection by members of the vibrion septique group. Such infection was formerly generally diagnosed as blackleg but has more recently been diagnosed as "malignant edema," "Ghon-Sachs bacillus infection," "specific gas phlegmon of hogs," or "Bradsot," depending on the location of the process. Hogs have probably never been shown to suffer from spontaneous blackleg infections.

The comparative rarity of oedematiens-group infections in animals, except perhaps in the horse, and the great rarity of serious invasion of animal tissue by B. welchii are to be noted.

In general many species of herbivorous mammals are subject to spontaneous anaerobic infection, both following and not following demonstrable wounds, and apparently the animals most susceptible are

ruminants, cattle and sheep, and possibly reindeer. Carnivores and man are subject to anaerobic infection only when wounded or when seriously debilitated by sickness.

All immunization work in connection with anaerobic diseases depends on a recognition of the group (see pp. 388, 389) affinities of the anaerobic organisms infecting the animals of the particular district in which immunization is proposed.

The factors concernng anaerobic infection that are of most interest, namely, the epidemiologic factors, are almost wholly unexplained, and their demonstration would prove a most valuable contribution to science and to agriculture.

I wish to make a plea for the world-wide study of these infections. They cost every agricultural country vast sums every year, and notable steps in the direction of immunization have shown that such immunization is feasible. To decide definitely the mode of infection in these diseases and the incidence of the group and specific entities involved would be of immense value to any country and to the world at large. The specificity of the toxins and of the aggressins of the different members in each group must be investigated. There are a hundred interesting immunologic and epidemiologic problems to be suggested. The proper investigation of the subject can be done only in a laboratory that is well equipped to handle anaerobes and that has the funds to employ a trained epidemiologist who knows something of veterinary pathology and can spend all his time in the field.

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THE EFFECT OF STREPTOCOCCUS HEMOLYTICUS INFECTION ON THE REACTION OF THE BLOOD OF RABBITS *

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It has been amply demonstrated by many workers using a wide variety of methods of examination that the reaction of the blood remains constant in health and is subject to slight actual variations in disease. Human blood is faintly alkaline and expressed in a physico-chemical sense has a hydrogen-ion concentration only slightly less than that of pure water. As determined by its dissociation into hydrogen and hydroxyl ions, pure water at 20 C. contains approximately 1-10,000,000 gm. of hydrogen ions to the liter and an equivalent amount of hydroxyl ions; in other words, pure water is 1-10,000,000 N acid and also 1-10,000,000 N alkaline. This reaction is conveniently expressed in terms of the hydrogen-ion concentration by the use of logarithmic exponents, such as 10^{-7} or as suggested by Sörensen [1] as P_H 7. When human blood is titrated by electrometric methods its reaction is found to be P_H 7.45, and the limits of variation to be well within P_H 7.0 and P_H 8.0. The neutral point P_H 7.0 is reached only in severe acidosis (as a terminal finding) and the limit of alkalinity P_H 8.0 only after the prolonged administration of alkalies. This precise regulation of the reaction of the blood constitutes one of the great constants of the body. The acid radicals produced through the oxidation of carbon, sulphur, and phosphorus in the food under normal conditions and in large part the acid radicals which arise through deranged metabolism in disease are either oxidized or neutralized in the body and then excreted or excreted unchanged. The fixed bases in the food and the ammonia arising from protein metabolism furnish bases for such neutralization, and the excretion of carbon dioxid by the lungs and of acid by the kidneys complete the mechanism. In addition the blood itself is able to take care of considerable quantities of either acid or alkali without change in its reaction through the buffer action

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1. Ergebn. d. Physiol., 1912, 12, p. 393.

of the carbonates and phosphates of its plasma. From the recent experiments of Bayliss,[2] it is probable that the proteins of the plasma play no perceptible part in the maintenance of neutrality between the limits of hydrogen-ion concentration possible in the living organism.

While it is thus seen that the reaction of the blood is delicately adjusted by a relatively efficient compensatory mechanism, nevertheless in pathologic conditions under which there may be an excessive production of normal acids as well as abnormal acids or a rapid excretion of bases or a defective excretion of acids, a breakdown in this mechanism occurs. Such a failure in the compensatory mechanism is called acidosis, and the general conception of this condition is that there occurs a diminution in the reserve supply of fixed bases in the blood and other tissues of the body, the physicochemical reaction of the blood remaining unchanged except in extreme conditions. With such a conception it has been definitely demonstrated that an acidosis occurs in diabetes, acute nephritis,[3] and in certain alimentary disturbances in children.[4] Many workers have reported acidosis in febrile conditions and in acute infections,[5] a situation which might be anticipated were there an associated injury to the kidneys. In very few cases of acute infections, however, has an actual increase in the hydrogen-ion concentration of the blood been reported and none in which an actual acidity, i. e., a concentration greater than P_H 7.0 was present.

In the recent epidemic of influenza, secondary systemic invasion by Streptococcus hemolyticus was frequently observed and in these cases was probably responsible for death. A common necropsy finding was edema of the brain and the so-called cloudy swelling of the parenchymatous organs, changes which have been explained on the basis of Fischer's theories on the action of body colloids as due to an acidosis. It is quite evident that the hydration capacity of the tissue colloids could not be increased through a simple depletion of plasma carbonate with no change in the actual reaction of the blood. since this increased capacity is assumed to be due to the presence of free acid. An acidosis of rather marked grade might therefore be expected in Streptococcus hemolyticus infections.

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[4] Howland, J., and Marriott, W. McK., Bull. Johns Hopkins Hosp., 1916, 27, p. 63.
[5] Friderica, L. S., and Olsen, Otto, Deutsch. Arch. f. klin. Med., 1912, 107, p. 236. Walker, I. C., and Frothingham, C., Arch. Int. Med., 1916, 18, p. 304.

In a recent paper Fleming [6] has laid great stress on the reaction of the blood and tissue fluids as an important part of the protective mechanism of the body both in resisting and overcoming infection. He states that "The normal reaction of the blood fluids corresponds to an N/35 alkaline solution" and he believes that this degree of alkalinity greatly restricts the growth of such bacteria as B. welchii. Almroth Wright [7] has demonstrated that B. welchii grows luxuriantly in blood serum which has been "neutralized" by the addition of 2 volumes of N/10 sulphuric acid to 7 volumes of serum, or blood serum which has been "acidified" by adding 3 volumes of N/10 sulphuric acid to 7 volumes of serum but that it does not grow at all in untreated blood serum. He believes that the fact that the avalanche-like invasion of the organism by the gas bacillus after a certain critical point in the infection has been reached may be due in part to the developing acidosis. In a later paper [8] Wright and Fleming have demonstrated that a similar condition obtains for the other organisms present in cases of gas gangrene, i. e., vibrion septique, Bacillus oedematiens, Bacillus fallax, Bacillus aerofoetidus of Weinberg. Attention should be called to the fact that there is an entire misconception of the chemistry of the blood in the statement that its normal reaction corresponds to an N/35 alkaline solution. The fact that the blood is equivalent to such solution in its power to neutralize acid must not be interpreted to mean that bacteria would encounter the same resistance to growth in both mediums. N/35 refers to the titratable alkalinity of the blood and not to its actual alkalinity. Furthermore the "neutralized" and "acidified" blood serum in the experiment of Wright and Fleming represent a degree of acidosis that has never been found during life and indeed could not occur.

Other investigators have conceived that the development of acidosis in an acute infection might represent an attempt at resistance in the production of a medium of a reaction unfavorable for bacterial proliferation. As seen from the previous considerations, such a change in the reaction of the blood is difficult of production and can only result after a breakdown in the compensatory mechanism; furthermore, the utmost limits of range of hydrogen-ion concentration possible in the living organism lie between 10^{-4} N and 10^{-10} N or P_H 4 and

[6] Brit. Jour. Surg., 1919, 7, p. 99.

[7] Lancet 1917, 1, p. 1.

[8] Lancet, 1918, 1, p. 205

P_H 10^2. Jones [9] found that a culture of virulent Streptococcus hemolyticus uniformly died out when the reaction of the medium reached P_H 5.11 or P_H 4.63 when ascitic fluid was added to the medium Other bacteria, such as B. typhosus, B. paratyphosus, and B. proteus, likewise show a definite limit of hydrogen-ion tolerance.

In order that the reaction of the body fluids might then resist the invading organism through the development of acidity it is necessary to demonstrate that the body fluids actually develop a hydrogen-ion concentration equal to the limit of the hydrogen-ion tolerance for the organism in question.

Streptococus hemolyticus was chosen for these experiments because its hydrogen-ion tolerance in mediums enriched by body protein is definitely known and also because it seemed possible that it would be quite a powerful agent in causing acidosis, not only through its toxic effect on body cells in general, but also because of its specific hemolyzing effect on the red blood corpuscles. As suggested to me by Dr. Le Count there is definite anatomical evidence for the occurrence of acidosis in streptococcus infections. In comparing the segments of brains cut by serial section after the usual hardening in formol solution, it has been noted for a number of years in this laboratory that with streptococcus infections the swelling and paleness of the brain has been quite like that of the brains of persons dying from uremia and diabetic coma, diseases in which an acidosis develops some time before death. Rabbits were chosen since they are quite susceptible to streptococcus infections and are large enough to withstand the repeated bleedings necessary for blood examinations. The determination of the presence of acidosis involved a consideration only of those methods for measuring an actual increase in the hydrogen-ion concentration of the blood. The usual tests for acidosis, such as increase in tolerance to sodium bicarbonate, lowering of the carbon dioxide of the alveolar air and of the blood, and increase in the output of ammonia in the urine, were discarded since they indicate only a depletion of fixed bases and give no information concerning the actual reaction of the blood. A reduction of the bicarbonate reserve of the blood may be associated with an increase, a decrease, or no change in the hydrogen-ion concentration. An exceedingly accurate measurement of the hydrogen-ion concentration of the blood may be obtained by means of the hydrogen electrode. However, if the hydrogen electrode is to be used for this purpose

[9] Jour. Infect. Dis., 1920, 26, p. 160.

the gaseous atmosphere with which the blood is brought into equilibrium must contain carbon dioxide at a tension equal to the alveolar carbon dioxide tension of the experimental animal. Such a measurement of the alveolar carbon dioxide tension in experimental animals is very difficult and the method is not practicable. Levy, Rowntree, and Marriott [10] have introduced a method of measuring the hydrogen-ion concentration of the blood by the use of indicators, which is simple, readily applicable to animal experiments of this nature, and sufficiently accurate to demonstrate changes that could have any significance for this work. It consists in determining the reaction of the dialysate of oxalated whole blood by the addition of a definite quantity of phenolsulphonephthalein and a comparison of the resulting quality of color with that of colored standard phosphate mixtures of known hydrogen-ion concentration.

REACTION OF THE BLOOD IN NORMAL RABBITS

Blood collected from the marginal ear vein of rabbits, oxalated, dialyzed through collodion sacs, and the hydrogen-ion concentration of the dialysate determined according to the method of Levy, Rowntree, and Marriott. Thirty determinations were made on eight normal rabbits at varying intervals during the day with the following results:

P_H of Oxalated Whole Blood	Number of Examinations
7.6	6
7.65	12
7.7	11
7.75	1

Thus of 30 examinations the reaction of the whole blood was found to range between P_H 7.6 and 7.75. Levy, Rowntree, and Marriott found the reaction of human blood under normal conditions to vary between P_H 7.4 and 7.6. The higher degree of alkalinity in rabbits (herbivora) is probably due to the fact that the diet is rich in free bases and finds confirmation in the normal alkaline reaction of the urine.

REACTION OF THE BLOOD IN STREPTOCOCCUS HEMOLYTICUS INFECTIONS

Strains of hemolytic streptococci isolated from spinal fluids, pleural fluids, and peritoneal fluids in human infections were used. From 2

[10] Arch. Int. Med., 1915, 16, p. 389.

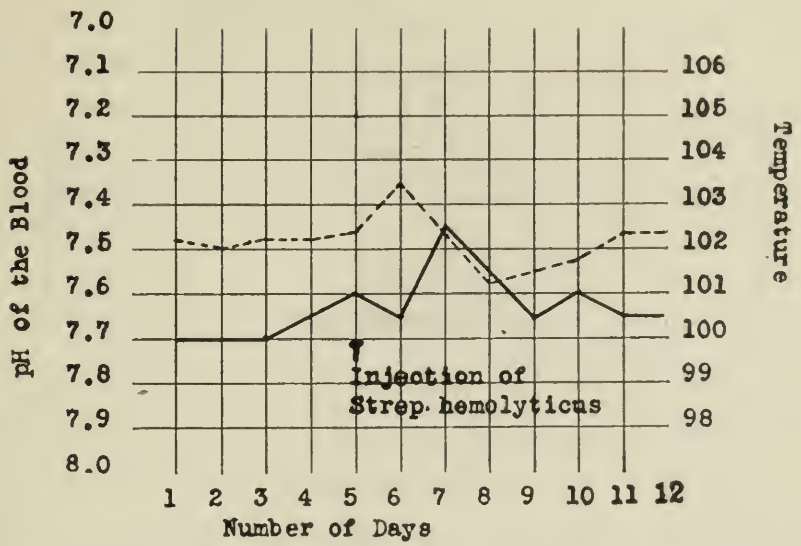

Chart 1 (Exper. 1).—Effect of intraperitoneal injection of 1 c c of a 24-hour culture of St. hemolyticus isolated from pleural fluid. The broken line indicates temperature, the continuous line is the curve of reaction of the blood.

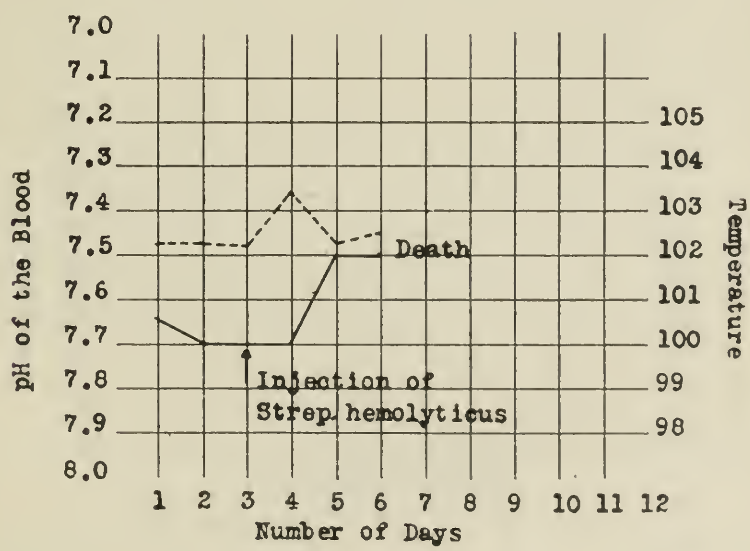

Chart 2 (Exper. 1).—Result of intraperitoneal injection of 2 c c of a 24-hour broth culture of St. hemolyticus isolated from the heart blood of a rabbit dying from a streptococcus infection. Death occurred 72 hours later. Necropsy revealed a dry plastic fibrinous general peritonitis. St. hemolyticus was isolated in pure culture from the peritoneal cavity and the heart blood. The broken line indicates temperature; the continuous line is the curve of reaction of the blood.

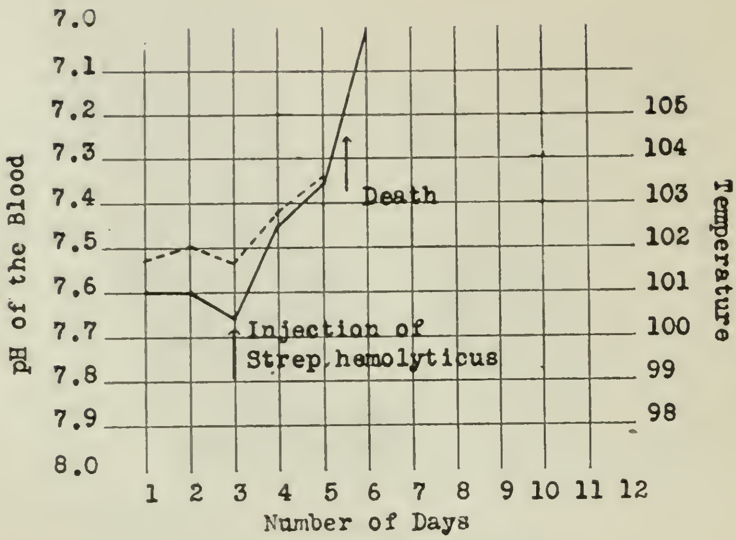

Chart 3 (Exper. 1).—Result of intraperitoneal injection of 2 c c of a 24-hour broth culture of St. hemolyticus isolated from the heart blood of a rabbit dying from a streptococcus infection. Death occurred 48 hours later. Necropsy revealed a general serofibrinous peritonitis. St. hemolyticus was isolated in pure culture from the peritoneal cavity and the heart blood. The marked degree of acidosis found in the blood after death may probably be explained by the fact that the excretion of acids by way of the lungs and kidneys ceases immediately at death while the production of acids through the metabolism of cells continues for varying periods thereafter. The broken line indicates temperature; the continuous line is the curve of reaction of the blood.

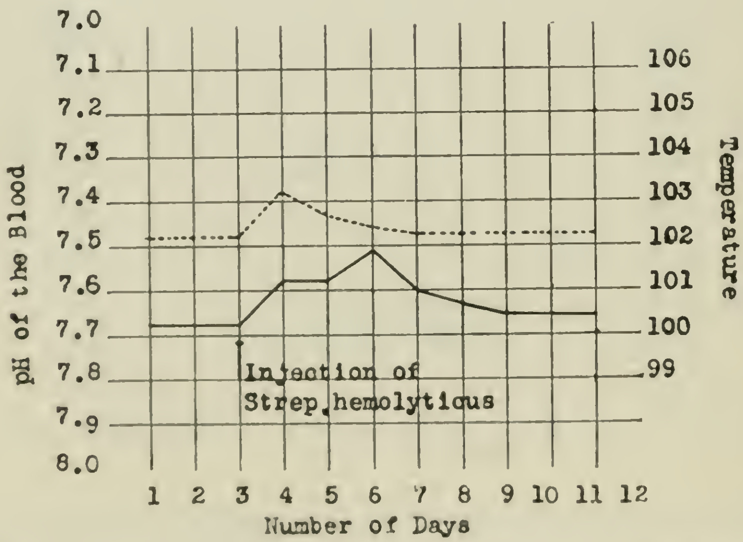

Chart 4 (Exper. 1).—Composite curve showing the effect on the temperature and reaction of the blood of rabbits following the intraperitoneal injection of from 2 to 4 c c of broth cultures of St. hemolyticus (16 experiments). An intraperitoneal injection of killed streptococci in amounts equal to those used for the other experiments produced practically no rise in temperature nor variation in the reaction of the blood greater than occurs under normal conditions with this method of determination. The broken line indicates temperature; the continuous line is the curve of reaction of the blood.

to 4 c c of a 24-hour broth culture of these strains was injected intraperitoneally and subsequent daily observations of the general condition, temperature, weight, and determinations of the reaction of the blood were made. The following curves are arranged to show the variations in the P_H of the blood during the course of the infections. The animals lost weight following inoculation, but the loss was not marked during the brief period of each experiment so the curves are not plotted.

DISCUSSION

The maximum acidity observed during life in any of the animals in the course of the experiments was P_H 7.3 and even after death the reaction of P_H 7.0 indicated an acidity equal to that of pure water. When it is recalled that Streptococcus hemolyticus can grow in medium enriched with serum until the acidity reaches P_H 4.63 it is evident that the development of acidosis per se in an acute infection can play no inhibitory rôle in the progress of the infection. As a change in the reaction of the blood toward the alkaline side is even more difficult of attainment due to the ready availability of acids in the body, it is likewise evident that for the reaction of the body fluids to play any part in the mechanism of resistance to bacterial infection the range of hydrogen-ion tolerance and of hydroxyl-ion tolerance of the specific organism in question must lie within the range found in the blood during life, i. e., between P_H 7.0 and P_H 8.0. There remains, of course, the possibility that the alteration in the reaction of the body fluids occurring during the course of the infection may in some way influence the fixation of antigens, the elaboration of specific immune bodies, or the activity of the leukocytes. These are points which are beyond the scope of the present investigation.

CONCLUSIONS

The blood of normal rabbits is slightly more alkaline than human blood.

Experimental Streptococcus hemolyticus infections in rabbits produce a relatively marked acidosis, the P_H of the blood varying from 7.65 which is normal, to 7.3 in an extreme case.

The development of acidosis in Streptococcus hemolyticus infections in rabbits does not in itself play any effectual rôle in the mechanism of resistance.

AGGLUTINATION IN INFLUENZA

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During the last epidemic in St. Louis in February, 1920, agglutination tests were made on serum from patients with influenza in the St. Louis Children's Hospital and the Barnes Hospital in order to determine (1) whether the serum of the patient agglutinates the influenza bacillus obtained from the same patient, (2) whether agglutination takes place with heterologous strains.

Studies of the agglutination reaction in influenza have not given uniform results. The first attempt to find specific antibodies in influenza serum was made in 1903 by Cantani [1] who examined three patients, two with acute influenza pneumonia, and their serum failed to cause agglutination; the serum of the third patient with uncomplicated influenza caused agglutination at 1 : 200. No further study was made until 1916, when Levinthal [2] found a strongly positive agglutination in 6 cases of uncomplicated influenza of which 3 were positive in a dilution of 1 : 400. 2 in 1 : 200 and 1 in 1 : 100. A seventh patient had a complicating pneumonia and caused agglutination 1 : 200. Levinthal emphasizes the diagnostic value of this test. I shall mention a few only of the studies that have been made recently.

Flemming [3] obtained positive agglutination in all of 21 cases but in low dilution. once at 1 : 1,000, twice at 1 : 128, three times at 1 : 64, in ten at 1 : 32, in four at 1 : 16, and in one at 1 : 8. Most of the patients were convalescent from 7 to 10 days after the onset of influenza. His control tests were all negative.

Duval and Harris [4] found agglutination in most instances, but not in a dilution above 1 : 80 and often as early as the third day of the disease. Normal blood reacted in a few instances but never above 1 : 20.

Martha Wollstein [5] examined serum from 11 patients during the second week of illness; 5 mild cases of bronchopneumonia gave positive results in a dilution of 1 : 100. Of 6 patients with uncomplicated influenza, 4 gave negative results and two positive, in dilutions of 1 : 40. The control serums sometimes gave a positive reaction in dilutions of 1 : 10. The tendency to spontaneous clumping, which was the chief difficulty, suggests that agglutination reactions with bacillus of Pfeiffer may not be satisfactory. Wollstein, like most of the authors who have worked with vaccinated patients, found that agglutinins are developed in the blood from 6 to 10 days after inoculation.

[1] Ztschr. f. Hyg. u. Infectionskrankh., 1903, 42, p. 505.

[2] Ztschr. f. Hyg. u. Infectionskrankh., 1918, 86, p. 1.

[3] Lancet, 1919, 1, p. 138.

[4] Jour. of Infect. Dis., 1919, 25, p. 384.

[5] Jour. Exper. Med., 1919, 30, p. 555.

From this short résumé it can be seen that all the authors mentioned found agglutinins for influenza bacilli in the blood of patients with influenza, present in greater or less strength and in accordance with the frequency of positive results more or less stress has been placed on the diagnostic and etiologic value of the test.

No studies on agglutination of homologous and heterologous strains by the serum of patients appear to have been made. Park [6] and his co-workers came to the conclusion that while specific agglutination with the homologous serum was obtained with the serum of immunized rabbits, each strain seemed to be individual in its immunologic action.

Small and Dickson [7] recently found characteristic agglutination with some strains so that influenza bacilli might be separated into four groups.

Passing to my own observations, it must first be mentioned that the antigen was prepared in the following way: 48 hour cultures grown on blood agar and originally obtained from the nose or throat by Dr. H. H. Bell and Dr. White were suspended in 0.8% salt solution. After thoroughly shaking, a homogeneous emulsion was usually obtained which did not show any evidence of self agglutination. Only in one instance was it necessary to discard the results on account of spontaneous clumping.

After 4 hours' incubation at 37 C. the tubes were put in the icebox and the result read 24 hours after mixtures were made.

Thirty strains were isolated from 30 patients during the first days after admission to the hospital and tested with serum from these patients during different stages of the disease. Of these, 11 (36%) gave a positive agglutination with their own bacilli, 6 in a dilution of 1 : 160; 4 in 1 : 80 and 1 in 1 : 20.

These serums were then tested with heterologous strains. With the exception of one strain, which gave an agglutination of 1 : 40 with three serums of other patients, there was found no cross agglutination in any serum tested. Each strain seemed to be individual in its immunologic reaction.

Five control serums from persons who gave no history of having had influenza were tried with different strains. In one instance an agglutination at 1 : 20 was obtained. In all other instances the result was negative.

[6] Jour. Am. Med. Assn., 1919, 73, p. 318.
[7] Jour. Infect. Dis., 1920, 26, p. 230.

The blood of 9 patients was examined during different stages of the disease; 8 showed a constantly negative result while in one instance the agglutination became positive in a dilution of 1 : 80 in the convalescent stage of the disease. Two strains were isolated from this patient and both gave the same result on agglutination.

RESULTS OF TESTS

No.	Day of Disease	Agglutination	Age	Remarks
1	6	1:160	41 years	Bronchopneumonia
2	11	1:160	35 years	Convalescent after uncomplicated influenza
3	11	1:80	38 years	Convalescent after uncomplicated influenza
4	11	1:80	25 years	Convalescent after uncomplicated influenza
5	10	1:160	68 years	Convalescent after uncomplicated influenza
6	8	1:160	5 years	Convalescent after uncomplicated influenza
7	1	1:80	8 years	Convalescent after uncomplicated influenza
8	14	1:160	10 years	Influenza with lobar pneumonia; fever during 13 days
9	7	1:160	2 years	Influenza with slight bronchitis
10	15	0	1 year	First afebrile day after light lobar pneumonia. Discharged on 19th day, recovered
	19	1:80	1 year	
11	8	1:20	13 months	Influenza with bronchopneumonia which disappeared 16 days after onset
	11	0	13 months	
	25	0	13 months	
12	13	0	27 years	Uncomplicated influenza
13	12	0	23 years	Convalescent after uncomplicated influenza
14	7	0	18 years	Uncomplicated influenza
15	10	0	30 years	Influenza with bronchopneumonia; fever during first 12 days
16	10	0	32 years	Convalescent after uncomplicated influenza
17	8	0	3 years	Influenza with fever during whole time in hospital
	11	0	3 years	
18	16	0	14 years	Influenza with bronchopneumonia. Fever during 31 days
	30	0	14 years	
19	9	0	2 years	Influenza with fever 10 days
20	13	0	4 years	Influenza with bronchopneumonia; delayed resolution; fever in hospital,
	27	0	4 years	
21	7	0	13 months	Influenza with bronchopneumonia and otitis media; fever 10 days
22	8	0	2 years	Influenza with bronchitis; fever 12 days
23	10	0	6 years	Influenza with chronic bronchopneumonia. Tuberculosis
	13	0	6 years	
24	8	0	7 years	Influenza with otitis media
25	7	0	6 years	Influenza with chronic bronchopneumonia
	10	0	6 years	
	24	0	6 years	
26	4	0	11 years	Bronchopneumonia in both lungs
	29	0	11 years	
27	5	0	4 years	Uncomplicated influenza
	9	0	4 years	
28	7	0	1½ years	Influenza with bronchopneumonia; fever during 7 days
29	13	0	5 years	Influenza with bronchopneumonia; lungs not clear at discharge from hospital
30	10	0	3 years	Influenza with bronchopneumonia

It is of interest that 3 strains of the influenza bacillus were isolated from the same plate made from the throat of patient 10. These strains were shown by Dr. Bell to be distinctly different by agglutination and absorption tests with monovalent immune serum. Only one of these

strains gave agglutination with the patient's serum. The strains isolated from Case 10 are represented by Nos. 25, 26, and 27, on Chart 1 of Dr. Bell's report [8] No. 25 gave agglutination.

During uncomplicated influenza the body does not always form antibodies against the influenza bacillus. It would have been desirable to have observed these patients after complete recovery, but after discharge from the hospital it was not possible to make the further examinations.

SUMMARY

Of 30 strains isolated from patients with influenza 11, or 36 per cent., gave agglutination with the patient's serum. Seven of these occurred in uncomplicated influenza, and 4 in influenza pneumonia. Of the remaining 19 negative cases, 10 had pneumonia.

Of 30 strains only one was agglutinated by heterologous serum.

[8] Jour. Infect. Dis., this issue.

RELATION OF DIFFERENT STRAINS OF INFLUENZA BACILLI AS SHOWN BY CROSS AGGLUTINATION AND ABSORPTION TESTS

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Great endeavor has been made to throw light on the obscure problems relative to the etiology of influenza. Investigation of this character was more general during the epidemic occurring early in 1920 than during the greater one of the previous year, although the opportunity to observe patients suffering from this malady lasted over a much briefer period. In association with each epidemic much interest has been taken in the small gram-negative bacillus described by Pfeiffer [1] as Bacillus influenzae. Little by little knowledge is accumulating which it is hoped will show what relation this organism may have to the disease.

In anticipation of the epidemic that occurred early in 1920, a study of the influenza bacillus was commenced late in October, 1919. A survey was made to determine the incidence of the bacillus in the upper respiratory tract of healthy medical students from different localities of the Middle West.

Great stress was laid on the technic in this survey. The swabs were prepared from fairly heavy copper wire with the terminal 15 mm. bent to an angle of approximately 135 degrees to facilitate swabbing high in the pharynx. Cultures made from the pharynx gave more positive results than those from the tonsillar regions. The advantage gained by swabbing the pharynx had been pointed out by Winchell and Stillman.[2]

The medium employed was meat infusion 2% agar to which 4% defibrinated rabbit blood was added. Slightly more or less blood made no appreciable difference in the degree of growth, while marked divergence showed less favorable results. Four per cent. seemed to be the mean point of maximum growth. Brown blood was made by heating the medium to approximately 80 C. sufficiently long to pro-

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[1] Ztschr f. Hyg. u. Infectionskrankh, 1893, 13, p. 357

[2] Jour. Exper. Med., 1919, 30, p. 497.

duce a distinct brown color without a trace of red. The most desirable hydrogen-ion concentration was throught to be P_H 7.3 or 7.4. Here again organisms that grew well bore considerable variation of reaction. Nevertheless, P_H 7.3 or 7.4 represented the mean point of alkalinity showing maximum growth. It is of interest that Avery [3] used a medium with a final P_H of 7.3 to 7.5, while Winchell and Stillman used medium with a P_H of 7.2 to 7.5. In the first part of my work red blood medium was used exclusively although later more brown blood medium was employed. Brown blood was preferred because the colonies grew more luxuriantly and were considered as characteristic to those experienced as the so-called dew-drop colonies on red blood agar.

Petri dishes were prepared and inoculated along one border from the sides of the swab by rolling it on the surface of the agar, and from this inoculated area the organisms were spread over the remaining surface of the plate with a platinum needle. The area of primary inoculation from the swab always showed a confluent growth, from which small gram-negative bacilli were demonstrated in every instance in a stained film. From 10-30 colonies were picked from the sparsely inoculated area in the endeavor to isolate the small gram-negative bacillus observed in the film. A Petri dish was divided into small squares by a glass-pencil. The colonies picked from the throat culture were inoculated into these squares and incubated 24 hours. This gave sufficient material from each colony picked to avoid losing it, and when colonies were crowded avoided contamination to a great extent. This method was fairly rapid. If the organism was not isolated from the first throat culture, subsequent cultures were made and in a few instances as many as 5 cultures were taken from the same throat. A series of 28 was studied. Failure to isolate the organism was recorded in one instance after 7 attempts, although in the stained film a few gram-negative bacilli were observed. These were accepted as influenza bacilli, if they failed to grow on meat infusion 2% agar of desirable hydrogen-ion concentration (to which 4% ascitic fluid was added) and if they grew well when 4% defibrinated blood was added.

Winchell and Stillman studied the incidence of the influenza bacillus in normal throats by reculturing once a month from December, 1918, to May, 1919, inclusive. The percentage of positive results varied from 13 to 51%.

[3] Jour. Amer. Med. Assn., 1918, 71, p. 2050.

Jordan[4] found that 10 of 13 strains of the influenza bacillus studied by him had the property of producing indol. By plating he found the organism in 40% of normal throats. However, by testing for indol in association with mixed cultures he believes that its presence was demonstrated in 80% of his series.

The bacillus of influenza was found in the mouths of 35.1% of all healthy men examined at Camp Funston during the autumn of 1918 by Opie[5] and his associates.

Lord[6] and his co-workers made cultures from the throats of 34 healthy Harvard students at an army training camp in order to find the bacillus of influenza and found 76% of the cultures were positive. They give a table showing the occurrence of the influenza bacillus in association with diseases other than influenza which shows that they were present in from 19 to 100% of cases in tuberculosis, measles, varicella, etc.

Park[7] points out the importance of careful selection of material for culture, good medium and long training in the study of colonies. Too great emphasis cannot be laid on his comment.

It must be remembered that the survey under consideration was made on the crest of an epidemic and merged into a study of the incidence of the influenza bacillus in the throats of influenza patients.

The bacillus of influenza was isolated from the throats of all patients from whose throats I made cultures, the material being selected from patients in the St. Louis Children's Hospital. For a time the work was carried on by Dr. Park J. White, and at the beginning of his work he obtained positive cultures in about 30%. This figure rose as experience and familiarity with the technic increased, until he also was able to isolate the organism from 100% of influenza patients. On recultivating the cases still accessible from which negative results had previously been obtained, he was able to isolate the organism in each instance.

Opie[8] and his associates, in the report on pneumonia following influenza at Camp Pike, stated that the influenza bacillus was invariably present in the upper respiratory tract of patients with influenza.

Park[7] states that Williams cultivated 278 cases for the influenza bacillus with these results:

Hospital cases	80% positive
Marines	100% positive
Home for Children	98% positive

Pritchitt and Stillman[9] studied the incidence of the influenza bacillus and the pneumococcus in association with influenza and pneumonia and found:

Normal	42% positive
Convalescent from influenza	46% positive
Uncomplicated influenza	83% positive
Influenza with bronchopneumonia	93% positive
Bronchopneumonia	100% positive
Lobar pneumonia	55% positive

4 Jour. Am. Med. Assn., 1919, 72, p. 1542.

5 Ibid., p. 108.

6 Ibid., p. 188.

7 Ibid., 1919, 73, p. 318.

8 Ibid., 72, p. 556.

9 Jour. Exper. Med., 1919, 29, p. 259.

Winchell and Stillman found 39% positive in an asylum for boys in which no influenza occurred; 38% were positive among 52 convalescents from influenza in a home for girls. Jordan[10] found that 64% of cases cultivated by him between October, 1918, and February, 1919, gave positive results.

No attempt is made to review the literature on the incidence of the influenza bacillus in the normal throat, its incidence in association with other diseases or in association with influenza. Sufficient is quoted, however, to show the variation in results obtained.

A small diplostreptococcus resembling those studied especially by Tunnicliff[11] was observed in a number of instances. No special study was made of this organism.

The morphology of the influenza bacillus varied considerably; still it may be stated that 3 main types were observed: The more usual type was chiefly composed of small bacilli, which were fairly uniform in size and contour. A few or many forms showing pleomorphism were usually present. Coccoid forms, bacilli with one end broader than the other, some large bacilli, and heavy polar staining were observed. The pleomorphism varied to some extent in degree and type, depending on the age of the culture and the medium employed. No leptothrix forms have been observed in these strains after between 4 and 6 months' cultivation on brown blood medium. The second type was a small bacillus which was uniform in size and contour showing no pleomorphism. A subdivision of this type was made; these strains showed long slender filaments or leptothrix forms which manifested greater or less capacity for division. The filaments were of uniform diameters. In certain instances the filaments completely disappeared and only short bacilli of uniform size and contour were observed, in other instances, however, filaments recurred. The filaments at times showed unilateral or bilateral depressions on the sides, which seemed to indicate an attempt at division. A third type is represented by fairly long slender bacilli with slightly pointed ends and at times slightly curved. A few moderately long forms were seen, but as a rule the size was rather uniform. No very short forms were observed. Two strains of this type were studied and showed another peculiarity referred to later.

After long cultivation little change in the organism and cultures was observed; however, the growth was more luxuriant. Most strains produced a grayish growth on brown blood agar and the so-called "dewdrop" colonies when transferred to red blood agar. A few strains

[10] Jour. Infect. Dis., 1919, 25, p. 28.

[11] Ibid., 1920, 26, p. 405.

produced a colorless growth on brown blood agar, although in several instances a slightly grayish color appeared in older cultures. All the strains required hemoglobin in the medium. No distinct and constant changes on the blood were produced by their growth. The organisms grew better aerobically and in fresh medium which was not dried.

Pfeiffer's "Bacillus influenzae" was gram-negative; the bacilli were small and forms in division resembled cocci. Long filaments were observed and considered as involution forms. The organism was nonmotile and noncapsulated. He later isolated very long forms from a child with bronchopneumonia following diphtheria, which he was unable to differentiate from the former organism and designated it as "Bacillus pseudo-influenzae."

Four years later Grassberger[12] studied the morphology of this organism and recorded short forms and filamentous forms. Sometimes the poles of the bacillus showed greater affinity for stain than the body. He believed that the age of the culture and reaction of the medium influenced morphologic changes.

Wollstein[13] divided her cultures into two chief types; short forms, showing pleomorphism and at times polar staining, and long forms showing a greater or less degree of division.

Galli Valerio[14] discussed involution forms of this organism. He mentioned ovoid forms, spherical forms, clubbed forms which are often in juxtaposition, forms showing irregular staining, and long forms. These changes were observed especially in old cultures.

In 5 cases in my series colonies were picked from the same plate which fulfilled the requirements of the influenza bacillus, but which, however, differed in morphology. These strains were investigated by agglutination and absorption tests in 3 instances.

Twenty-seven rabbits were immunized and bled when the preliminary titration of the serum was satisfactory, that is, above 1,600.

The strains selected were from normal throats in 5 cases and from the throats of influenza patients in 22. Nos. 2, 3, 4, 5 and 10 came from throats of students who had never had influenza. Nos. 18 and 29 were isolated from influenza suspects. The rest were isolated from typical influenza cases and as a rule early in the development of the disease, although in a few instances pneumonia was developing.

The initial dose proved fatal to several rabbits within an hour or two. Parker[15] and others have demonstrated the toxicity of these organisms for laboratory animals. The most toxic strain was No. 4.

[12] Ztschr. f. Hyg. u. Infektionskrankh, 1897, 25, p. 453.

[13] Jour. Exper. Med., 1915, 22, p. 445.

[14] Rév. méd. de la Suisse Rom., 1919, 6, p. 265

[15] Jour. Immunol., 1919, 4, p. 331.

This organism was isolated from a normal throat of a student who never had influenza, fully 6 weeks before the onset of the epidemic in this vicinity.

Each serum was titrated with 36 organisms in dilutions of 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200 and 1:6,400. In a few instances other dilutions were added. Control emulsions were always incubated. Emulsions of organisms were made by washing a 24-hour growth from the surface of a brow blood agar slant or Petri dish with salt solution. Nos. 29, 26, 31 and 32 gave difficulty. The first of these showed auto-agglutination in the control. In the remaining 3 the growth was cohesive and resisted breaking µp into a fine suspension. The last two organisms are the ones described under the discussion of the morphology as type 3. This resistance to emulsification was constant; satisfactory results were obtained by using an 18 or 24 hour-growth on several freshly prepared moist plates. This suspension was shaken for 20 minutes and centrifuged sufficiently long to throw down the larger particles, leaving the finer ones in suspension. The same technic applied to No. 29 was also satisfactory. The agglutinating tubes were incubated at 53 C. during 18 or 20 hours and read macroscopically.

It should be observed on chart 1 that cross agglutination occurred frequently in low dilutions and in a few instances in fairly high dilutions. Some serums have a wider latitude of agglutination than others. Some organisms have a greater susceptibility to agglutination than others. Perhaps this is explained by group relationship.

Absorption tests were employed in those instances which showed cross agglutination in a dilution of 1:800 or above. A 24-hour growth on 3 to 6 Petri dishes was washed off the agar with a dilution of 1:25 of the serum to be absorbed. The mixture was incubated at 53 C. for 4 hours, shaken well at 15 minute intervals, and then left to stand over night in the refrigerator. The clear serum from the top was pipeted off with a capillary pipet. The absorbed serum represented a dilution of 1:25. Some of the unabsorbed serum of the original dilution was kept in the incubator 4 hours at 53 C. and in the refrigerator over night; it was titrated with its own organism as a control and the result is recorded on chart 2 following the series of absorption tests for which it serves as control.

It should be observed that these organisms vary markedly in their capacity to absorb specific agglutinin from a foreign serum.

TABLE 1

Agglutination by Monovalent Immune Serum with Different Strains of B. Influenzae

Numbers Indicate the Highest Dilution in which Agglutination was Present.

Immune serum.........	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	
Organisms	Dewey	Esserman	Ferguson	Dock	Lasersohn	Parsons	B. Ouslander	R. Ouslander	Bell	Shindlewolf	Gerhart	No. 198	Burdsley	No. 257	No. 219	Cotton	Whitaker	Bunyard	No. 226	No. 240	Hirst 1	Hirst 2	Fiorella 1	Fiorella 2	Budrovitch 1	Budrovitch 2	Budrovitch 3	
1 Dewey............	3200	50	400	100	25	25	0	100	200	0	0	50	50	100	200	100	200	25	200	0	....	25	....	0	0	200	0	1
2 Esserman.........	200	7000	50	200	0	50	200	100	0	0	50	0	100	400	50	0	0	25	0	100	....	0	....	25	0	400	0	2
3 Ferguson..........	100	100	5000	800	400	200	100	200	200	50	400	400	400	800	100	25	50	200	0	800	....	200	....	50	25	0	0	3
4 Dock...	800	100	400	3200	0	50	25	50	200	0	50	50	100	800	100	25	25	25	50	50	....	25	....	50	100	0	0	4
5 Lasersohn.........	200	50	400	800	2000	800	50	50	800	100	400	200	400	800	400	100	50	200	200	800	....	800	....	800	800	200	0	5
6 Parsons...........	50	0	800	200	2000	3200	25	100	100	0	100	50	400	200	50	25	100	0	50	800	....	50	....	100	800	50	0	6
7 B. Ouslander......	200	100	100	400	25	200	3200	6400	400	25	1600	100	400	400	100	50	200	100	100	200	....	0	....	50	25	0	0	7
8 R. Ouslander......	100	200	200	200	25	100	3200	6400	200	0	800	50	800	200	100	50	400	25	100	100	....	0	....	100	50	0	0	8
9 Bell..............	100	0	200	100	100	50	800	800	3200	25	800	800	400	50	50	0	0	400	1600	800	....	1600	....	50	50	0	0	9
10 Shindlewolf........	0	0	0	0	0	0	0	0	0	3200	0	0	0	0	0	50	50	0	0	0	....	0	....	0	0	25	0	10
11 Gerhart...........	100	100	200	400	50	100	100	400	800	25	2400	800	800	800	200	25	100	25	800	100	....	200	....	400	800	800	0	11
12 No. 198............	0	0	50	25	25	25	0	0	200	25	0	3200	0	0	50	25	0	0	800	25	....	0	....	0	0	0	0	12
13 Burdsley...........	0	0	100	25	200	50	25	25	0	50	100	200	6400	50	0	0	0	400	100	200	....	25	....	25	400	100	0	13
14 No. 257............	50	50	50	200	0	0	25	25	800	0	800	800	800	6400	100	50	0	0	0	400	....	25	....	50	25	25	0	14
15 No. 219............	200	50	800	800	400	200	100	100	200	25	100	400	400	800	6400	800	200	50	50	1600	....	1600	....	800	200	25	0	15
16 Cotton............	200	0	200	800	200	50	25	50	200	50	400	800	100	800	400	6400	100	25	50	800	....	25	....	50	400	50	0	16
17 Whitaker..........	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6400	0	0	0	....	0	....	0	0	0	0	17
18 Bunyard...........	25	0	200	0	100	0	50	0	200	0	0	0	0	0	0	0	0	3200	0	50	....	25	....	0	0	0	0	18
19 No. 226............	50	0	50	200	50	25	100	200	800	100	200	800	400	50	0	0	50	200	6400	200	....	400	....	200	400	25	0	19
20 No. 240............	200	50	100	50	800	100	50	50	100	50	100	200	200	800	800	25	400	200	800	6400	....	400	....	50	800	100	0	20
21 Hirst 1............	0	0	0	0	0	0	0	0	200	0	0	200	0	0	0	0	0	50	0	0	3200	1600	....	0	0	0	0	21
22 Hirst 2............	0	0	0	25	0	0	0	0	200	0	0	200	0	0	0	0	0	25	0	0	3200	1600	....	0	0	0	0	22
23 Fiorella 1..........	25	100	200	100	25	100	50	50	0	0	400	50	200	400	25	0	0	0	50	100	....	25	6400	3200	0	25	0	23
24 Fiorella 2..........	25	100	400	200	100	50	100	100	0	0	200	100	100	400	25	50	0	25	50	100	....	25	3200	3200	25	0	0	24
25 Budrovitch 1.......	25	200	800	100	800	400	0	25	200	25	400	200	400	400	0	50	25	200	50	400	....	200	....	100	3200	400	0	25
26 Budrovitch 2.......	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	....	0	....	0	0	3200	0	26
27 Budrovitch 3.......	0	0	0	0	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	....	0	....	0	0	0	6400	27
28 Fitzporter.........	0	0	0	0	200	0	0	0	0	25	0	0	0	0	0	0	25	0	0	0	....	0	....	0	50	0	0	28
29 Smith.	0	50	0	100	200	200	100	200	800	200	800	400	800	800	400	0	400	0	400	0	....	200	....	400	200	400	0	29
30 Meyer.............	0	200	200	200	200	100	200	400	800	25	800	200	200	0	25	0	200	200	200	0	....	25	....	25	25	0	0	30
31 Gebhard...........	0	100	0	400	0	50	0	0	0	0	25	0	0	0	50	25	0	0	0	50	....	0	....	25	0	0	0	31
32 Solinski...........	0	100	0	0	0	50	0	0	400	200	25	0	0	0	100	50	0	0	50	0	....	0	....	0	400	0	0	32
33 V. Brooks..........	25	0	0	50	0	0	0	0	0	0	0	0	0	0	0	0	800	0	0	0	....	0	....	0	0	0	0	33
34 Stolte.............	100	50	200	200	400	50	0	50	50	0	200	100	200	400	0	0	50	0	50	200	....	25	....	800	400	25	0	34
35 Brady.............	200	25	800	200	200	400	100	100	800	50	400	400	800	200	50	25	0	1600	1600	800	....	800	....	100	400	0	0	35
36 Fenton............	50	0	0	200	0	0	25	100	0	0	0	0	0	0	0	0	0	25	0	0	....	0	...	25	0	0	0	36
Immune serum.........	1	2	,3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	

Where absorption occurred these tests were repeated two and occasionally three times to cause complete absorption if possible. The results were confirmative of the first observations. In a few instances the titer of the control was lower than the original titer of the serum. Diluted serum of several days' standing was occasionally used and the titer of this was often diminished. This made no difference, however, as the control and the absorbed serum were of the same dilution.

When a serum was absorbed by a foreign organism, this organism absorbed all, part, or none of the agglutinins for other foreign strains so tested. This was subject to considerable variation.

It should be observed that B. Ouslander and R. Ouslander were identical strains. These were isolated from two children of the same family suffering from influenza. Hirst 1 and Hirst 2 strains isolated from the same plate, showing marked difference in morphology, were nevertheless identical strains. Fiorella 1 and 2 were isolated from the same plate and showed a marked difference in morphology; these were identical strains. On the contrary, Budrovich 1, 2 and 3, isolated from the same plate and showing marked difference in morphology, were proved to be distinct types, as shown by these tests.

The serum of No. 5, agglutinated organisms 5 and 6 in equally high dilution. By absorption tests it was shown that these organisms were not identical. No 25 had the capacity to absorb nearly all the specific agglutinins from serum of No. 5. This test was repeated 3 times, using a greater excess of organisms, with uniform result. This relation was not reciprocal. They were not identical strains. No grouping into types can be recognized on these charts. However, certain strains are closer related than others and can be divided into subtypes to a limited extent. For example, Nos. 5 and 6, also 9 and 19, show considerable cross agglutination. Many other strains show a close relation to these subtypes, which adds difficulty to separating them into distinct subgroups.

Two additional strains were studied later and not included in the charts. One was isolated from the lung of an adult with lobar pneumonia caused by the pneumococcus type 2. The second was isolated from a child suffering from bronchopneumonia with abscess formation following measles. Streptococcus hemolyticus and Micrococcus aureus were also isolated from the areas of consolidation. These strains resembled the organism included in the table and in no instance were they agglutinated by dilutions above 1:800. No absorption

TABLE 2

Cross Agglutination After Absorption Tests

Immune Serum for Organism	Absorbed by Organism	Titrated with Organism	Titer
No. 1			
Dewey	Dewey	Dewey	0
Dewey	Dewey	Dock	0
Dewey	Dock	Dock	0
Dewey	Dock	Dewey	800
Dewey	Unabsorbed (control)	Dewey	1600
No. 3			
Ferguson	Ferguson	Ferguson	0
Ferguson	Ferguson	Parsons	0
Ferguson	Ferguson	Lasersohn	0
Ferguson	Ferguson	219	0
Ferguson	Ferguson	Budrovitch 1	0
Ferguson	Ferguson	Bell	0
Ferguson	Parsons	Parsons	0
Ferguson	Parsons	Ferguson	3200
Ferguson	Lasersohn	Lasersohn	0
Ferguson	Lasersohn	Ferguson	3200
Ferguson	219	219	0
Ferguson	219	Ferguson	3200
Ferguson	Brady	Brady	0
Ferguson	Brady	Ferguson	3200
Ferguson	Budrovitch 1	Budrovitch 1	0
Ferguson	Budrovitch 1	Ferguson	3200
Ferguson	Unabsorbed (control)	Ferguson	3200
No. 4			
Dock	Dock	Dock	0
Dock	Dock	Ferguson	0
Dock	Dock	Lasersohn	0
Dock	Dock	219	0
Dock	Dock	Cotton	0
Dock	Ferguson	Ferguson	0
Dock	Ferguson	Dock	800
Dock	Lasersohn	Lasersohn	0
Dock	Lasersohn	Dock	800
Dock	219	219	0
Dock	219	Dock	800
Dock	Cotton	Cotton	0
Dock	Cotton	Dock	400
Dock	Unabsorbed (control)	Dock	1600
No. 5			
Lasersohn	Lasersohn	Lasersohn	0
Lasersohn	Lasersohn	Parsons	0
Lasersohn	Lasersohn	240	0
Lasersohn	Lasersohn	Budrovitch 1	0
Lasersohn	Lasersohn	Stolte	0
Lasersohn	Lasersohn	Ferguson	0
Lasersohn	219	219	0
Lasersohn	219	Lasersohn	400
Lasersohn	Parsons	Parsons	0
Lasersohn	Parsons	Lasersohn	400
Lasersohn	240	240	0
Lasersohn	240	Lasersohn	800
Lasersohn	Budrovitch 1	Budrovitch 1	0
Lasersohn	Budrovitch 1	Lasersohn	50
Lasersohn	Budrovitch 1	240	400
Lasersohn	Unabsorbed (control)	Lasersohn	1600
No. 6			
Parsons	Parsons	Parsons	0
Parsons	Parsons	Lasersohn	0
Parsons	Lasersohn	Lasersohn	0
Parsons	Lasersohn	Parsons	3200
Parsons	Unabsorbed (control)	Parsons	3200
No. 7			
B. Ouslander	B. Ouslander	B. Ouslander	0
B. Ouslander	B. Ouslander	Brady	0
B. Ouslander	R. Ouslander	R. Ouslander	0
B. Ouslander	R. Ouslander	B. Ouslander	0
B. Ouslander	R. Ouslander	Brady	0
B. Ouslander	Bell	Bell	0
B. Ouslander	Bell	B Ouslander	800
B. Ouslander	Brady	Brady	0
B. Ouslander	Brady	B. Ouslander	1200
B. Ouslander	Brady	R. Ouslander	800
B. Ouslanedr	Unabsorbed (control)	B. Ouslander	1600
No. 8			
R. Ouslander	R. Ouslander	R. Ouslander	0
R. Ouslander	R. Ouslander	B. Ouslander	0
R. Ouslander	B. Ouslander	B. Ouslander	0
R. Ouslander	B. Ouslander	R. Ouslander	0
R. Ouslander	Unabsorbed (control)	R. Ouslander	1600
No. 9			
Bell	Bell	Bell	0
Bell	Bell	Lasersohn	0
Bell	Bell	Gerhart	0
Bell	Bell	257	0
Bell	Bell	226	0
Bell	Bell	Brady	50
Bell	Bell	Meyer	0
Bell	Lasersohn	Lasersohn	0
Bell	Lasersohn	Bell	2400
Bell	Lasersohn	Brady	800
Bell	Gerhart	Gerhart	0
Bell	Gerhart	Bell	1600
Bell	226	226	0
Bell	226	Bell	800
Bell	226	Meyer	0
Bell	Brady	Brady	0
Bell	Brady	Bell	800
Bell	Brady	Lasersohn	0
Bell	Meyer	Meyer	0
Bell	Meyer	Bell	1600
Bell	Meyer	226	400
Bell	257	257	0
Bell	257	Bell	1600
Bell	Unabsorbed (control)	Bell	2400
No. 11			
Gerhart	Gerhart	Gerhart	0
Gerhart	Gerhart	B. Ouslander	0
Gerhart	Gerhart	R. Ouslander	0
Gerhart	Gerhart	Bell	0
Gerhart	Gerhart	257	0
Gerhart	Gerhart	Meyer	0
Gerhart	B. Ouslander	B. Ouslander	0
Gerhart	B. Ouslander	Gerhart	400
Gerhart	R. Ouslander	R. Ouslander	0
Gerhart	R. Ouslander	Gerhart	800
Gerhart	Meyer	Meyer	0
Gerhart	Meyer	Gerhart	100
Gerhart	Unabsorbed (control)	Gerhart	1600
Gerhart	Bell	Bell	0
Gerhart	Bell	Gerhart	1600
Gerhart	257	257	0
Gerhart	257	Gerhart	3200
Gerhart	Unabsorbed (control)	Gerhart	3200
No. 12			
198	198	198	0
198	198	B. Ouslander	0
198	198	Bell	0

TABLE 2—*Continued*

CROSS AGGLUTINATION AFTER ABSORPTION TESTS

Immune Serum for Organism	Absorbed by Organism	Titrated with Organism	Titer	Immune Serum for Organism	Absorbed by Organism	Titrated with Organism	Titer
B. Ouslander	B. Ouslander	R. Ouslander	0	No. 16			
B. Ouelander	B. Ouslander	Bell	0	Cotton	Cotton	Cotton	0
198	198	257	0	Cotton	Cotton	219	0
198	198	Cotton	0	Cotton	219	219	0
198	198	Gerhart	0	Cotton	219	Cotton	800
198	198	226	0	Cotton	219	R. Ouslander	200
198	Bell	Bell	0	Cotton	Unabsorbed	Cotton	800
198	Bell	198	2400		(control)		
198	B. Ouslander	B. Ouslander	0	No. 17			
198	B. Ouslander	198	1600	Whitaker	Whitaker	Whitaker	0
198	Gerhart	Gerhart	0	Whitaker	Whitaker	V. Brooks	0
198	Gerhart	198	2400	Whitaker	V. Brooks	V. Brooks	0
198	257	257	0	Whitaker	V. Brooks	Whitaker	3200
198	257	198	2400	Whitaker	Unabsorbed	Whitaker	3200
198	Cotton	Cotton	0		(control)		
198	Cotton	198	1600	No. 18			
198	226	226	0	Bunyard	Bunyard	Bunyard	0
198	226	198	2400	Bunyard	Bunyard	Brady	0
198	Unabsorbed	198	3200	Bunyard	Brady	Brady	0
	(control)			Bunyard	Brady'	Bunyard	1600
No. 13				Bunyard	Unabsorbed	Bunyard	1600
Burdsley	Burdsley	Burdsley	0		(control)		
Burdsley	Burdsley	Gerhart	0	No. 19			
Burdsley	Burdsley	257	0	226	226	226	0
Burdsley	Burdsley	R. Ouslander	0	226	226	Bell	0
Burdsley	Burdsley	Brady	0	226	226	Gerhart	0
Burdsley	Gerhart	Gerhart	0	226	226	198	0
Burdsley	Gerhart	Burdsley	2400	226	226	240	0
Burdsley	257	257	0	226	226	Brady	0
Burdsley	257	Burdsley	2400	226	Bell	Bell	0
Burdsley	R. Ouslander	R. Ouslander	0	226	Bell	226	800
Burdsley	R. Ouslander	Burdsley	2400	226	Gerhart	Gerhart	0
Burdsley	Brady	Brady	0	226	Gerhart	226	1600
Burdsley	Brady	Burdsley	2400	226	Gerhart	240	50
Burdsley	Unabsorbed	Burdsley	2400	226	198	198	0
	(control)			226	198	226	1600
No. 14				226	198	Brady	0
257	257	257	0	226	240	240	0
257	257	Ferguson	0	226	240	226	1600
257	257	Dock	0	226	240	Gerhart	50
257	257	Lasersohn	0	226	Brady	Brady	0
257	257	Gerhart	0	226	Brady	226	1600
257	257	219	0	226	Brady	198	100
257	257	Cotton	0	226	Unabsorbed	226	1600
257	257	240	0		(control)		
257	Ferguson	Ferguson	0	No. 20			
257	Ferguson	257	3200	240	240	240	0
257	Ferguson	Dock	200	240	240	Ferguson	0
257	Dock	Dock	0	240	240	Lasersohn	0
257	Dock	257	4800	240	240	Parsons	0
257	Lasersohn	Lasersohn	0	240	240	Bell	0
257	Lasersohn	257	4800	240	240	Cotton	0
257	Gerhart	Gerhart	0	240	240	219	0
257	Gerhart	257	4800	240	240	Brady	0
257	219	219	0	240	Ferguson	Ferguson	0
257	219	257	3200	240	Ferguson	240	3200
257	Cotton	Cotton	0	240	Lasersohn	Lasersohn	0
257	Cotton	257	4800	240	Lasersohn	240	800
257	240	240	0	240	Parsons	Parsons	0
257	240	257	4800	240	Parsons	240	100
257	Unabsorbed	257	4800	240	Bell	Bell	0
	(control)			240	Bell	240	1600
No. 15				240	Cotton	Cotton	0
219	219	219	0	240	Cotton	240	1600
219	219	240	0	240	219	219	0
219	240	240	0	240	219	240	1600
219	240	219	3200	240	Brady	Brady	0
219	240	Lasersohn	200	240	Brady	240	1600
219	Unabsorbed	219	3200	240	Unabsorbed	240	3200
	(control)				(control)		

TABLE 2—*Continued*

Cross Agglutination After Absorption Tests

Immune Serum for Organism	Absorbed by Organism	Titrated with Organism	Titer
No. 21			
Hirst 1	Hirst 1	Hirst 1	0
Hirst 1	Hirst 1	Hirst 2	0
Hirst 1	Hirst 2	Hirst 2	0
Hirst 1	Hirst 2	Hirst 1	0
Hirst 1	Unabsorbed (control)	Hirst 1	1600
No. 22			
Hirst 2	Hirst 2	Hirst 2	0
Hirst 2	Hirst 2	Hirst 1	0
Hirst 2	Hirst 2	Bell	0
Hirst 2	Hirst 2	Lasersohn	0
Hirst 2	Hirst 2	219	0
Hirst 2	Hirst 2	Brady	0
Hirst 2	Hirst 1	Hirst 1	0
Hirst 2	Hirst 1	Hirst 2	0
Hirst 2	Bell	Bell	0
Hirst 2	Bell	Hirst 2	3200
Hirst 2	Lasersohn	Lasersohn	0
Hirst 2	Lasersohn	Hirst 2	3200
Hirst 2	219	219	0
Hirst 2	219	Hirst 2	3200
Hirst 2	Brady	Brady	0
Hirst 2	Brady	Hirst 2	3200
Hirst 2	Unabsorbed (control)	Hirst 2	3200
No. 23			
Fiorella 1	Fiorella 1	Fiorella 1	0
Fiorella 1	Fiorella 1	Fiorella 2	0
Fiorella 1	Fiorella 2	Fiorella 2	0
Fiorella 1	Fiorella 2	Fiorella 1	0
Fiorella 1	Unabsorbed (control)	Fiorella 1	1600
No. 24			
Fiorella 2	Fiorella 2	Fiorella 2	0
Fiorella 2	Fiorella 2	Lasersohn	0
Fiorella 2	Fiorella 2	Stolte	200
Fiorella 2	Fiorella 2	219	0
Fiorella 2	Fiorella 2	Fiorella 1	0
Fiorella 2	Lasersohn	Lasersohn	0
Fiorella 2	Lasersohn	Fiorella 2	1600
Fiorella 2	Lasersohn	Fiorella 1	1600
Fiorella 2	Stolte	Stolte	0
Fiorella 2	Stolte	Fiorella 2	1600
Fiorella 2	Stolte	219	400
Fiorella 2	219	219	0
Fiorella 2	219	Fiorella 2	1600
Fiorella 2	Fiorella 1	Fiorella 1	0
Fiorella 2	Fiorella 1	Fiorella 2	0
Fiorella 2	Fiorella 1	Lasersohn	0
Fiorella 2	Unabsorbed (control)	Fiorella 2	1600
No. 25			
Budrovitch 1	Budrovitch 1	Budrovitch 1	0
Budrovitch 1	Budrovitch 1	Lasersohn	0
Budrovitch 1	Budrovitch 1	Parsons	0
Budrovitch 1	Budrovitch 1	240	0
Budrovitch 1	Budrovitch 1	Budrovitch 2	0
Budrovitch 1	Lasersohn	Lasersohn	0
Budrovitch 1	Lasersohn	Budrovitch 1	800
Budrovitch 1	Lasersohn	240	400
Budrovitch 1	Parsons	Parsons	0
Budrovitch 1	Parsons	Budrovitch 1	800
Budrovitch 1	240	240	0
Budrovitch 1	240	Budrovitch 1	400
Budrovitch 1	Budrovitch 2	Budrovitch 1	1600
Budrovitch 1	Budrovitch 3	Budrovitch 1	1600
Budrovitch 1	Unabsorbed (control)	Budrovitch 1	1600
No. 26			
Budrovitch 2	Budrovitch 2	Budrovitch 2	0
Budrovitch 2	Budrovitch 2	B. Ouslander	0
Budrovitch 2	Budrovitch 2	B. Ouslander	0
Budrovitch 2	Budrovitch 2	Gerhart	50
Budrovitch 2	B. Ouslander	B. Ouslander	0
Budrovitch 2	B. Outlanedr	Budrovitch 2	3200
Budrovitch 2	Gerhart	Gerhart	0
Budrovitch 2	Gerhart	Budrovitch 2	3200
Budrovitch 2	Budrovitch 1	Budrovitch 2	3200
Budrovitch 2	Budrovitch 3	Budrovitch 2	3200
Budrovitch 2	Unabsorbed (control)	Budrovitch 2	3200
No. 27			
Budrovitch 3	Budrovitch 1	Budrovitch 3	3200
Budrovitch 3	Budrovitch 2	Budrovitch 3	3200
Budrovitch 3	Unabsorbed (control)	Budrovitch 3	3200

tests were made. Finally all strains were tested for agglutination with normal rabbit serum. No agglutination occurred in dilutions 1 : 25 or above.

Small and Dixon [16] studied 10 strains of the influenza bacillus by agglutination and absorption tests. They concluded that this organism can be divided into groups by these methods and that they encountered 4 groups.

Park, Wollstein, and Fleming and Clemenger [17] have been unable to divide the influenza bacillus into groups by agglutination. Valentine

[16] Jour. Infect. Dis., 1920, 26, p. 230.

[17] Lancet, 1919, 2, p. 869.

and Cooper,[18] after employing agglutination and absorption tests, believe that they are dealing with a heterogenous group or organisms with subgroups, and that identical strains are encountered.

CONCLUSIONS

The influenza bacillus represents a heterogenous group of organisms as shown by agglutination and absorption tests. Identical strains occur.

No differentiation can be made by these methods between the organisms isolated from normal healthy throats within two months preceding the epidemic and those isolated from the throats of influenza patients.

A person may carry in the throat three different strains of this organism at the same time.

The morphology alone is unreliable as a means of dividing this group of organisms into subgroups.

[18] Jour. of Immunol., 1919, 4, p. 359.

COMPLEMENT FIXATION IN INFLUENZA WITH B. INFLUENZAE ANTIGENS

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During the past two or three years some doubt has arisen regarding the part played by B. influenzae as an etiologic agent in influenza. It is agreed that the organism is almost universally present in the upper respiratory secretions of patients with the disease, and also that there are large numbers of bacillus carriers both in healthy individuals and in those suffering from other respiratory infections. From this widespread distribution of the influenza bacillus, therefore, has arisen the question whether the organisms found in influenza patients are present as infecting parasites, or merely as relatively harmless saprophytes. If parasitic, they may be the cause of the disease or may be present as secondary invaders, although the latter seems somewhat less likely. Since one of the evidences of infection by an organism is the production of specific antibodies, some effort has been made to determine the presence of specific antibodies to the influenza bacillus in the serums of influenza patients. The presence of such specific substances would supply indirect evidence of the etiologic relation of the organism to influenza. This article gives the results of a study of serums from influenza patients by the complement fixation test using several strains of B. influenzae as antigens.

Several investigators have recently studied the complement fixing substances in influenza serums, but all have not obtained uniform results.

The largest series of cases was reported by Rapoport,[1] who found 54.5% of positive fixation in 295 serums from patients convalescing from influenzal pneumonia, using influenza bacillus antigens. Some serums were positive 41 days after convalescence began, but most of the positive reactions were obtained on patients convalescent 3 to 4 days. Durand[2] with an influenza bacillus antigen obtained negative fixations in 5 cases of uncomplicated influenza during convalescence, and 2 questionably positive reactions in 4 patients with pneumonia. Four other cases in which B. influenzae was found in the nasopharynx gave 2 positive and 1 questionable fixation. Kolmer, Trist and Yagle,[3] found 45 to

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[1] Jour. Am. Med. Assn., 1919, 72 p 633

[2] Riforma Med., 1919, 35, p 458.

[3] Jour. Infect Dis., 1919, 24, p. 583

51% of positive fixations in 31 cases of convalescent influenza with Pfeiffer bacillus antigens, while 38% of the same serums were positive with both the hemolytic streptococcus, and M. catarrhalis antigens in addition. Reactions using staphylococcus and a pseudodiphtheria bacillus antigen were negative except one serum which reacted with the staphylococcus. Nine healthy controls gave negative fixation tests with all antigens. Howell and Anderson[4] obtained only 20% of positive fixations in 253 tests in 59 cases of influenza by the use of B. influenzae antigens, while they obtained many more positive results on serums from the same patients using as antigen certain strains of the viridans group of streptococci.

Gay and Harris,[5] using a polyvalent influenza antigen, obtained positive fixation in only one of 29 acute cases of influenza, but in 40% of 25 vaccinated patients from 2 to 37 days after the last injection. The serum of rabbits immunized with mixed cultures of B. influenzae gave fixing antibodies in high dilutions. Wollstein[6] examined 5 mild cases and 7 complicated by pneumonia, and obtained positive fixations in all and negative reactions in 4 normal adults. The cases were examined from the sixth to the twenty-third day of the disease, and stronger reactions were obtained as convalescence advanced. Eight different strains of Pfeiffer's bacillus were used as antigen, and some serums reacted with only one strain while other serums gave fixations with all strains tested. In 19 persons who had recovered from influenza from 1 to 4 months previously, irregular results were obtained, but some serums showed the presence of fixing antibodies. Rabbits immunized with 10 strains of influenza bacilli gave positive fixations with both homologous and heterologous strains. Fry and Lundie,[7] using a salt solution extract of one strain of B. influenzae, found 6 of 8 cases of influenza gave positive fixation reactions, while 32 controls showed one positive and 5 weakly positive or doubtful reactions.

In this paper are given the results of complement fixation tests in children and adults with influenza pneumonia, using several strains of B. influenzae as antigens.

METHODS

The strains of B. influenzae used were isolated by Dr. H. H. Bell and their source and characteristics described by him.[8] In preparation of the antigens, a 24-hour growth on blood-agar plates was washed off with salt solution to form an emulsion of moderate opacity. This was heated to 65 C. for one hour, and titrated to determine its anticomplementary properties. Each antigen was then diluted with salt solution so that half the largest amount, which was not at all anticomplementary, was contained in 0.1 c c and this antigen dose used in all tests. Usually a dilution of 4 or 5 times was required.

The patients' serums were inactivated for 30 minutes at 56 C. and 0.1 c c, 0.05 c c, and 0.01 c c used for each fixation test.

4 Jour. Infect. Dis., 1919, 25, p. 1.

5 Jour. Infect. Dis., 1919, 25, p. 414.

6 Jour. Exper. Med., 1919, 30, p. 555.

7 Lancet, 1920, 198 p. 368.

8 Jour. Infect. Dis., 1920.

The hemolytic system consisted of two units of complement with 0.5 c c of 1% suspension of sheep corpuscles sensitized with 2 units of hemolysin. The antigen, serum and complement were incubated 1 hour after the addition of the sensitized cells. The total volume of each test was 2.5 c c, and the readings made after standing over night in the icebox. Details of the method used may be found elsewhere.[9] The usual controls of serum without antigen and of twice the amount of antigen used were always included.

The considerable dilution of antigen required to overcome its anticomplementary power may have interfered in certain cases with its antigenic properties. However, since all antigens except one gave fixations with some serums, they were probably antigenic in the amounts used. No attempt was made to prepare a permanent antigen that could be used throughout the entire series of tests. The tests were carried out at various times and on each occasion the antigens were prepared in the same manner so that it is likely that the results of tests made at different times are for the most part comparable.

All possible cross-fixations could not be done on account of the lack of sufficient serum from every case. Each serum was tested with from 4 to 12 antigens and in some cases the tests were repeated with another specimen of serum taken at a later date. In order to control to a certain extent the specificity of the antigens, all serums were also tested with a human tubercle bacillus antigen and all gave negative results.

Twenty-one of the cases tested were uncomplicated, and had typical clinical symptoms with fever and leukopenia. In 14, bronchopneumonia of varying severity prolonged the illness but was fatal in only one instance. The blood in all cases, except the fatal one, was obtained after the temperature had become normal, usually in the second week after the onset of the first symptoms. All were patients in the Barnes and St. Louis Children's Hospitals during the epidemic of the early months of 1920. Organisms morphologically and culturally resembling B. influenzae were isolated from the sputum or the nasopharyngeal secretions of a number of the patients by Dr. H. H. Bell, Dr. Park J. White, and Dr. A. M. Chesney.

On account of the wide prevalence of the disease and the almost certain occurrence of many unrecognized mild infections, it was difficult to secure many suitable control serums from persons known to have escaped the disease. Four children and 9 adults (medical stu-

[9] Cooke, J. V.: Jour. Infect. Dis., 1919, 25, p. 452.

dents), none of whom had had any clinical symptoms of influenza, were selected as examples of probably uninfected persons and their serums used as controls.

In tabulating the results of the tests, a division has been made into several groups. Children under 6 years are shown in table 1, while the older children are given in table 2. This separation was made on account of an observation on complement fixation for tuberculosis in children.[10] Here it was found that tuberculous children under 6 years of age gave a much smaller proportion of positive results than the older children, while the percentage of the latter giving positive reactions was quite similar to that found in adults. In table 3, the tests on adults with influenza are collected, and in

TABLE 1

Complement Fixation Tests in Influenza in Children under 6 Years

	Case Number										
	1*	2*	3*	4*	5	6*	7	8	9	10*	11
Day of disease	8	7	21	7	7	12	4	8	11	8	15
Age in years	1†	1†	1†	2	2	2	3	3	4	4	4
Antigens:											
4. Cotton	..	0	++	0	..	..	0	0	..	0	
5. B. Ouslander	..	..	..	..	..	..	++				
6. 198	+	0	..	0	0	0	0	0	++	..	++
12. Lasersohn	+	±	++	0	0	0	0	0	0	0	0
13. Ferguson	0	++	+	0	0	0	++	+	++	0	++
14. Stolte	0	..	0	0	0	0	0	0	0	0	+
15. 257	..	..	0	++	0	..	..	0	..	0	+
16. Parsons	0	..	..	..	0	0	..	..	0	..	++

* B. influenzae isolated from pharynx.
† Influenza with pneumonia.

TABLE 2

Complement Fixation Tests in Influenza in Children 6 to 14 Years

	Case Number												
	12*	13	14*	15*	16*	17	18	19*	20*	21	22	23*	24*
Day of disease	7	14	7	7	14	6	12	16	14	30	28	12	30
Age in years	6†	6†	6†	6	7†	8	9†	9	10†	10	11†	13†	13†
Antigens:													
1. Essermann	++	..	..	++	..	..	..	..	..	..	..	++	
2. Smith	0	..	..	0	..	..	..	..	..	..	..		
3. 223	++	..	..	++	..	..	..	..	..	..	..	++	
4. Cotton	++	..	±	±	±	+	++	..	..	..	0	±	
5. B. Ouslander	++	..	++	++	++	..	..	..	..	..	..	++	0
6. 198	++	..	++	++	++	..	++	++	++	+	++	++	±
7. 240	++	..	++	±	++	..	..	..	..	..	..	++	
8. Bell	++	..	..	++	..	..	..	..	..	..	0	++	++
9. R. Ouslander	++	..	..	++	..	..	..	..	..	..	..	++	
10. 226	++	..	..	++	..	..	..	..	..	..	++	+	0
11. Dewey	++	..	..	++	..	.	..	..	..	..	..	±	
12. Lasersohn	..	++	++	..	..	±	++	0	++	+	0	..	0
13. Ferguson	..	++	++	..	..	+	++	++	++	0	++	..	++
14. Stolte	..	0	++	..	..	++	++	±	0	0	0	..	0
15. 257	..	..	++	..	..	+	++	0	..	..	0	..	0
16. Parsons	..	++	0	..	0	..	..	++	0	0	0	..	++

* B. influenzae isolated from pharynx.
† Influenza with pneumonia.

[10] Cocke, J. V.: Am. Jour. Dis. Child. In press.

TABLE 3

Complement Fixation Tests in Influenza — Adults

	Case Number										
	25	26*	27*	28*	29*	30*	31*	32	33*	34*	35*
Day of disease	30	13	13	10	14	10	10†	7†	14	10	10
Antigens:											
1. Essermann	..	..	++	..	..	++					
2. Smith	..	..	0	..	..	0					
3. 223	..	..	++	..	..	++					
4. Cotton	±	0	±	..	±	±	±	++	++	..	+
5. B. Auslander	..	++	++	++	..	++	++	..	..	++	++
6. 198	..	..	++	..	0	++	++	0	++		
7. 240	..	..	++	++	..	±	++	..	..	++	
8. Bell	..	..	++	..	..	++	..	..	..	..	++
9. R. Auslander	..	..	++	++							
10. 226	..	..	++	..	..	++	..	..	..	++	++
11. Dewey	..	..	++	++	..	++	..	..	..	..	±
12. Lasersohn	++	0	..	..	0	..	±	±	+		
13. Ferguson	++	0	..	..	++	..	±	±	++		
14. Stolte	±	0	..	..	..	..	0				
15. 257	0	++	..	..	..	..	0				
16. Parsons	0	..	..	..	..	..	0				

* B. influenzae isolated from sputum or from pharynx.
† Influenza with pneumonia.

TABLE 4

Complement Fixation Tests on Children and Adults Without Clinical Influenza

	Case Number												
	36	37	38	39	40	41	42	43	44	45	46*	47	48
Age in years	¼	3	5	8	A	A	A	A	A	A	A	A	A
Antigens:													
1. Essermann	..	0	0	..	..	..	0	..	..	..	0	..	0
2. Smith	..	0	..	..	0								
3. 223	0	0	0	..	..	..	0	..	..	..	0	..	0
4. Cotton	..	0	0	0	0	0	0	0	0	0	0	0	0
5. B. Auslander	0	..	0	0	..	..	..	..	..	0			
6. 198	..	0	0	0	0	0	0	0	++	±	0	0	0
7. 240	..	..	..	0	0								
8. Bell	..	..	..	..	..	0	0	0	++	++	++	++	++
9. R. Auslander	..	..	..	0	0	0	..	..	++	..	..	+	
10. 226	..	..	..	..	..	0	0	0	0	0	0	0	0
11. Dewey	0	..	0	..	0	..	..	0					
12. Lasersohn	0	0	0	0	0	0	0	0	0	0	0	0	0
13. Ferguson	0	±	0	0	0	0	±	0	0	0	++	0	++
14. Stolte	0	..	..	..	..	0	0	0	0	0	0	0	++
15. 257	..	..	..	..	..	0	0	0	0	0	0	0	0
16. Parsons	0	..	..	..	..	0	±	++	±	++	++	++	++

* B. influenzae isolated from pharynx.

table 4, the control tests. In indicating the strength of fixation found in the different serums, "++" shows complete fixation with 0.5 c c or less of serum; "+," complete fixation with 0.1 c c only, and "±," partial fixation with 0.1 c c. A "O" means no fixation with 0.1 c c and a blank indicates that the test was not made.

Eleven younger children, between 1 and 4 years old were tested with 8 strains of B. influenzae as antigen (table 1). While fixation was noted in all but 3 of the serums, with one or more antigens, the results were irregular. There was no uniform fixation with any of

the serums nor was any antigen constant in its fixing properties. Of the 59 tests, only 17 were positive, 6 of these being weak reactions.

With the older children and adults (tables 2 and 3) the presence of fixing bodies was much more evident, although in these also some irregularity was noted. Certain antigens fixed well with almost all cases tested, while other strains failed to give fixation with some of the serums. No order could be detected in this irregularity nor could any grouping of the antigens be made, since some antigens that gave good fixation with some of the serums, failed to fix in other instances in which other antigens had given positive results. In all 162 tests were made on 24 serums in these 2 groups using 16 strains of B. influenzae, and more than 60% of the reactions were positive. All serums tested gave fixation with two or more of the antigens, and only one of the antigens failed to give fixation with any of the serums. The patients with pneumonia as a rule gave good fixation, but in this group no better results were noted than those that did not develop this complication.

The controls included 13 cases (table 4) and of the 120 tests done, 16% were positive. These positive reactions were obtained in 6 of the serums with 6 of the antigens, one of the positive tests being given by the serum of an apparently healthy carrier. The explanation of the positive reactions obtained in certain of the controls is not clear, but the possibility of a previous unrecognized infection in these persons is difficult to exclude. By comparing the tables, however, there is a striking difference noted in the number of positive reactions obtained in the groups studied. Many more positive reactions were found in the older children and adults with influenza than in the group of younger children, while the control group showed the smallest number of positive reactions.

CONCLUSIONS

Complement fixing antibodies can be demonstrated in the serum of a considerable number of older children and adults convalescent from influenza by the use of B. influenzae antigens. These antibodies are much less constantly found in children from 1 to 5 years of age. No definite antigenic relationship could be detected between the 16 strains of B. influenzae with the serums tested. The results indicate that the influenza bacillus is pathogenic and infects many, if not all, patients with influenza. The complement fixation test cannot furnish sufficient evidence, however, to justify the conclusion that B. influenzae is the sole etiologic agent in influenza.

AN IMPROVED METHOD FOR THE PRODUCTION OF ANTIMENINGOCOCCIC AND OTHER SERUMS

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Most of those who have had to do with the preparation of antibacterial serum have experienced the long time required for immunization and loss of animals usually associated with the preparation of such serums. The usual period assumed to be required for the immunization of horses with meningococci and other bacteria was from 4 to 8 months until Flexner and Amoss [1] published their notes on the rapid production of antidysenteric serum based on the earlier work of Fornet and Müller,[2] Bonhoff and Tsuzuki [3] and especially Gay [4] and his colleagues, in which it was shown that by the repeated injection of antigenic materials at short intervals a quicker and more exalted response in antibody formation was produced. The method used by these various workers consisted in its essential details in the injection of the antigenic substances usually for 3 successive days with a variable period of resting. Previous to the application of these earlier results by Flexner and Amoss to production of serum on a large scale, it had been the usual practice in the production of antibacterial serum to inject horses with cultures of bacteria of different periods of growth and manner of culture, oftentimes containing autolysates or soluble bacterial products. The injections were given by various routes—subcutaneously, intramuscularly and intravenously—but the frequency of severe reactions, when the intravenous method was used, greatly lessened the employment of that method. Flexner and Amoss immunized their horses for the production of antidysenteric serum by 3 successive intravenous injections of living cultures or toxin with intervening rest periods of 7 days. They state that a suitable serum for therapeutic use in man could be prepared in about 10 weeks.

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[1] Jour. Exper. Med., 1915, 21, p. 515.

[2] Ztschr. f. biol. Techn. u. Method., 1908, 1, p. 201.

[3] Ztschr. f. Immunitätsforsch., 4, p. 180. Tsuzuki, M., ibid., p. 194.

[4] Gay and Fitzgerald: University of California Publications in Pathology, 1911-14, 2, 77. Gay, F. P., Ergebn. d. Immunitatsforsch., exper. Therap. Bakteriol. u. Hyg., 1914, 1, p. 231. Loke, E., University of California Publications in Pathology, 1911-14, 11, p. 91.

Amoss and Wollstein [5] applied the same methods to the production of antimeningococcic serum, but quickly encountered the difficulties of other workers in the severe and even fatal reactions of their horses. They endeavored to overcome this drawback by the use of the so-called desensitizing injection as described by Briot and Dopter.[6]

As used by Amoss and Wollstein, the desensitizing injection, consisting of $\frac{1}{20}$ to $\frac{1}{10}$ of a 24-hour agar slant culture was administered intravenously 2 hours before the full dose was given. They state that by the use of the desensitizing injection the severe reactions are avoided and that a satisfactory serum may be produced in 8 to 12 weeks instead of the much longer period required by other methods of immunization.

We had been using for some time the intravenous method injection but had been much troubled by the severe and even fatal reactions, so that when the work of Amoss and Wollstein appeared, the methods advised by them were given a trial.

It has been found that the time required for immunization was reduced by the method of 3 successive intravenous injections with a resting period of 7 days, but that the severe and even fatal reactions in the horses were not prevented by the use of a desensitizing dose. It was also found that it was not a satisfactory procedure to attempt to regulate the dose by the temperature curve as they suggested when many horses were under treatment.

As the demands for antimeningitic serum by the military forces were great and insistent, it was quickly realized that some method must be devised by which valuable animals producing a high titer serum could be prevented from dying and that the period required for immunization could be reduced even below that stated by Amoss and Wollstein.

Severe reactions were more frequently encountered among the horses used in the production of antimeningococcic serum than those used for the production of antipneumococcic serum, and the time required for immunization was appreciably longer, but severe and even fatal reactions occurred also in antipneumococcic horses.

As there were a considerable number of horses already producing serum of a high titer immunized by various procedures, it appeared more important to determine immediately, if possible, means by which severe reactions in these animals could be prevented.

[5] Jour. Exper. Med., 1916, 23, p. 403.
[6] Compt. rend. Soc. de biol., 1910. 69, p. 174.

Accordingly various modifications were made in the manner of growing the meningococci, preparing the emulsion for injection and method of injection. Any one who has witnessed the beginning, development and termination of a severe or fatal reaction in a horse following the injection of meningococci will have been struck by the similarity of the symptoms to those of anaphylaxis in the smaller animals, such as the guinea-pig, and it was not an unnatural assumption that the reaction in the horse was a manifestation of anaphylaxis or hypersensitiveness to the meningococci or its products, especially as the reaction is more frequently encountered later than the third or fourth course of injection, according to the Amoss-Wollstein scheme, or after the third or fourth week of treatment by other plans of immunization. This is, however, not invariably true as our records show a number of animals that had severe reactions, even followed by death, in some instances on the first day of treatment and a larger number during the first week of treatment.

Before going further with the discussion of the cause of the reaction and methods for prevention, I shall give a brief description of the reaction.

The reaction generally appears within 10 minutes and often in less than 5 minutes after an intravenous injection of meningococci or other bacteria. The first symptoms are rapid and shallow breathing, restlessness, sweating and general weakness.. The heart beat is increased but not in proportion to the respiratory difficulties. In the milder type of reaction the respiratory rate and character gradually improve and in 6 or 8 hours the animal returns to normal. In the severer reactions, the symptoms mentioned become aggravated, especially the difficulties of breathing and general weakness, so that the animal makes violent efforts to take in sufficient oxygen by the employment of all the accessory respiratory muscles. The horse finally becomes so weak as not to be able to stand and either falls or lies down, and is unable to rise. The temperature remains about normal. When death occurs it is apparently due to respiratory failure. Postmortem shows nothing distinctive; the right heart is full; the lungs are not distended or emphysematous; but the abdominal blood vessels appear distended with blood. Death has occurred following the first injection given, and in a number of instances has taken place within 5 minutes of the injection. If the symptoms do not appear within an hour after the injection, no reaction will

follow. In our experience with about 100 horses observed for the reaction, it has had no relation to the number of the injection in a series, the series of injection, the rapidity with which the injection was made or whether the injection consisted of killed or live meningococci, but only when the injection was given intravenously.

In addition to the general reaction described, most horses show an elevation of temperature running from 1 to 3 C. after an intravenous injection of meningococci, and animals may show a rise of as much as 3 degrees and no general reaction.

As stated, we made use of the desensitizing injection suggested by Dopter, but early found that by no modification of its use could we surely prevent the development of general and fatal reactions. We then began to question whether the reactions were a manifestation of hypersensitiveness to the meningococci or their products and in a short time became satisfied that such was not the case; but that the symptoms were due to the action of a poison present in the injected material. That this is true in some instances at least is shown by the development of severe reactions following the first injection ever given the animal, or during the first series of injections. Furthermore, the theory was supported by the fact that in the reactions could be prevented by the procedure, which I shall describe, and by the production of a similar reaction in rabbits after a single and first intravenous injection. The latter part of the work will be reported in detail in a subsequent paper.

As soon as it was realized that the reactions were caused by the action of some toxic substance in the material injected, efforts were directed to its removal and were early rewarded with success.

In going over each step in the preparation of antigen, we found that it was the custom, chiefly because of its convenience, for the bacteriologic laboratory to prepare the antigen for the day's injection as early as possible, and that it was the custom for the veterinary department to make the injections at a time most convenient for the other work to be done. In this way the injections usually were made within an hour after the preparation of the antigen, but that in some instances several hours elapsed. It was further found that reactions were more frequent and more severe when a considerable period had elapsed between the making of the antigen and the injection.

All injections of meningococci and other bacterial antigens were now made as soon after their preparation as possible, in no case

longer than 30 minutes and usually not more than 10 minutes after preparation. This reduced the frequency of the reactions, but did not entirely eliminate them. At this point some work under way on rabbits gave us a valuable lead. If the antigen were prepared in the usual way and injected intravenously into rabbits, the rabbit frequently died within a short period, but if the suspension of bacteria were centrifuged, washed once and resuspended, the injection of this washed suspension of bacteria would be without ill effect on the rabbit.

Accordingly, the antigen for the horses was prepared in a similar way and it was soon found that the use of a washed suspension of meningococci, given immediately after preparation, resulted in the elimination of all severe reactions, and of practically all general reactions except an occasional mild one, which usually could be attributed to some definite fault.

The meningococcus antigen, as now used, is prepared by growing meningococci for 18 to 24 hours at 37 C. on glucose serum agar in large test tubes. About 35 strains, embracing the various types of meningococci, are used. Approximately 10 c c of normal salt solution are added to each of half of the cultures and the growth washed off; this suspension is then added, 8 or 10 c c to each remaining tube and the organisms suspended in the fluid. It is then centrifuged for about 1 hour, or until the bacteria are closely packed and the supernatant fluid entirely removed, the bacteria are resuspended in salt solution, strained through sterile, fine meshed gauze, and by comparison with a standard turbidity scale the number of bacteria are adjusted to approximately 5,000 million per c c. The antigen thus prepared is injected immediately, using a small needle and making the injection slowly. The volume injected never exceeds 25 c c. When dead cultures are to be used, the bacteria are killed after the emulsion has been strained. The meningococci and pneumococci are killed by heating at 53 to 55 C. for 30 minutes.

Antigens from pneumococci are prepared by inoculating 2 liter bottles of dextrose broth, with a culture which has been passed through mice each week, growing at 37 C. for 16 to 20 hours, and the bacteria collected from this broth culture by passing through a Sharples' centrifuge. The pneumococci are suspended in salt solution, centrifuged, the supernatant fluid removed, washed once and a standard suspension of 5,000 million per c c made. For other bacterial antigens the same procedure is followed.

The points to be noted carefully are: young cultures, washing the suspension of bacteria, careful straining of the emulsion to remove large clumps of bacteria and particles of agar, prompt injection after washing, and slow injection by means of a small needle.

Most of those who have made use of the intravenous method of inoculation for the production of antimeningococcic serum have begun with the injection of very small amounts of antigen. Amoss and Wollstein used 0.1 c c of an agar slant suspended in 15 c c of salt solution: this was equivalent to about $\frac{1}{20}$ of an agar slant. We had begun with 0.1 c c of a polyvalent antigen and increased the amount 0.1 c c at each injection. We found this method would in time cause the development of antibodies in satisfactory amounts, but sought for a plan by which the immunization could be more rapidly brought about, and found that by the use of killed cultures this end could be attained. The scheme of treatment finally found to give the quickest and most satisfactory results was to begin with 0.5 c c of a killed washed polyvalent suspension of meningococci; the amount was increased 0.25 c c each day for 4 days with a resting period of 3 days, when the treatment was resumed, starting with the amount given at the last injection of the previous series. In the second series the dose was increased 0.5 c c and in subsequent series 1 c c at each dose. It was usually found that after the fourth series the antibodies were present in measurable amounts. After the fourth series of washed killed meningococci, the injection of live organisms was begun, the initial dose of washed live bacteria being 1 c c of an approximately 5,000 million per c c emulsion. Four daily injections were given with a resting period of 3 days between each series. The dose was increased at each injection from 0.5 to 1 c c.

A test bleeding was made after the third series of injections of live organisms so that such adjustments might be made in the composition of the antigen as would be most likely to produce a well balanced serum of equal valency to the various groups of meningococci.

For the other antibacterial serums, such as antipneumococcus and antistreptococcus serum, the same scheme was followed with such modifications as were desirable.

We have used the methods described for the immunization of over 200 horses and have not lost a single horse from acute death following the injection, nor have we had what might be called a severe reaction in any horse. Horses have died from the development of cardiac lesions, particularly lesions of the heart valves, but we

have not lost a horse used in the production of antimeningococcus serum for more than two years. Furthermore, these horses remain in good condition and the meningococcus horses are remarked as being in the best condition of all the horses used for the production of biologic products.

The results obtained in two groups of horses, one against meningococci and one against pneumococci, are given in order that the value of the plan as outlined may be the better appreciated.

In the meningococcus group there were 18 horses, whose treatment was begun at approximately the same time. The average period required before these horses produced a serum which would pass the requirements of the Hygienic Laboratory was 50.1 days; the shortest time for any of the groups was 44 days and the longest was 79. None of these horses had a severe reaction and of course none died from an injection.

In the pneumococcus group there were 49 horses whose treatment was begun about the same time. The average time required to immunize these horses to produce a serum to pass the requirements of the Hygienic Laboratory was 46 days; the shortest period was 34 days, of which there were not less than 7 in the group; the longest was 56 days.

It is quite possible that if all of the horses in both groups had been tested earlier that a number would have been ready for production before the fact was actually determined; but the showing is sufficient to demonstrate the great saving of the method in time and animals.

During the period of greatest demand for antimeningococcic serum, it became important that the interval between bleedings be reduced to the shortest time possible consistent with the maintenance of a high titer for the serum and good condition of the horses. We found that after the horses had reached the production point, there was no difficulty in maintaining them so, and even in increasing the titer of their serum by a single course of injections of antigen between bleedings. In this way we were able to bleed the horses for a time on an average of once in 16 days. We were able to increase the yield of serum by taking 2 bleedings, each of 6,000 c c, 48 hours apart, the first bleeding was taken 6 days after the last injection. By this means the percentage yield of serum to total amount of blood drawn was much increased over that from a single large bleeding. The

horses stood this intensive treatment well and at the end, without exception, the titer of the serum of all horses had increased.

It is now over two years since some of these horses were started on the production of antimeningococcic serum, and they are in good condition and producing a well balanced serum of a titer much higher than that required by the U. S. Public Health Service.

SUMMARY

A potent and well balanced antimeningococcic serum can be quickly and safely produced from the horse by the use of the method described.

The method involves the use of an antigen, given intravenously, prepared from washed, killed cultures of meningococci.

The severe and even fatal reactions so frequently encountered in the horse after intravenous injections of bacteria are not a manifestation of anaphylaxis, but are due to the presence of a toxic substance in the material injected.

This toxic material can be removed by centrifugation and washing, and its removal apparently does not reduce the antigenic properties of the emulsion.

A reaction similar to that observed in horses can be produced in rabbits by a first injection of an emulsion of unwashed meningococci; this reaction in the rabbit can be avoided by the use of an emulsion of washed meningococci.

The reaction has no relation to the number of the injection in a series, the series of injection, the rate of injection, or whether live or dead organisms are given, but occurs when the injection is intravenous and unwashed cultures are used.

Points to be noted carefully in the preparation of the antigen are: young cultures, washing the bacterial emulsion, straining the emulsion to remove large clumps and particles of mediums, prompt injection after washing, and slow injection.

Horses can be quickly brought to a production point by the use of washed killed cultures followed at a later period by washed live cultures.

The scheme of injection provides for 4 successive injections with a resting period of 3 days. The treatment is begun with killed cultures, changing to live organisms after the fourth series of injections.

The average time required to immunize satisfactorily a group of 18 horses to meningococci was 50.1 days; the shortest time was 44 days and the longest was 79 days.

The average time required to immunize a group of 49 horses to pneumococci was 46 days; the shortest was 34 days, and the longest 56 days.

The method is generally applicable to the production of bacterial antiserum.

CONCLUSIONS

The method reduces the period required to immunize horses for the production of antimeningococcic and other bacterial antiserums.

The severe and fatal general reactions following the intravenous injection of bacterial emulsions are due to a toxic substance in the emulsion and are not anaphylactic reactions.

This toxic substance can be almost entirely removed by washing the bacteria.

THE EFFECT OF ROENTGEN RAY AND THORIUM X ON PNEUMOCOCCUS AND STREPTOCOCCUS INFECTIONS IN MICE

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In many of the acute infectious diseases, e. g., typhoid fever, measles, and influenza, leukopenia is a characteristic blood change and of some diagnostic value. These diseases are frequently complicated by what is supposed to be a secondary or concurrent infection with pneumococci and streptococci so that the question has arisen whether the diseases themselves are not caused by organisms of the pneumococcus or streptococcus group. There may be two important sources of leukopenia, the first and probably more permanent and serious one being injury or destruction of the hematopoietic organs, such as can be produced experimentally by the use of benzene, the roentgen ray, and certain radioactive preparations, and the other which may only be a precursor to a leukocytosis may be the introduction of foreign or bacterial proteins [1] intravenously. In view of the conception of the intimate relationship between the leukocyte and antibody formation in combating acute infections, and having found that even a profound injury or destruction of the hematopoietic organs by means of benzene, roentgen ray and thorium X had no appreciable effect on the progress of experimental tuberculosis,[2] it seemed desirable to study the effect of the roentgen ray and thorium X on pneumococcus and streptococcus infections.

Kellert[3] noted no effect of the roentgen ray on experimental tuberculosis in the guinea-pig, but his animals seemed to be more susceptible to spontaneous acute infections after being exposed to the ray. The effect of benzene, roentgen ray and thorium X on antibody formation has been studied by Hektoen,[4] who found that these preparations all produced a marked leukopenia with grave lesions in the marrow and that benzene in rabbits greatly reduced the production of specific precipitin and lysin for sheep blood; and that exposure of white rats, and dogs and rabbits to the roentgen ray restrains the formation of specific

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[1] Wells, Clifford W., Jour. Infect. Dis., 1917, 20, p. 219.

[2] Corper, H. J., Amer. Rev. Tuberc., 1918, 2, p. 587.

[3] Kellert, Ellis: Jour. Med. Res., 1918, 39, p. 93.

Jour. Infect. Dis., 1916, 19, p. 69; 1915, 17, p. 415; 1918, 22, p. 28.

lysin for sheep blood to a marked degree. Hektoen and Corper[5] found that thorium X might reduce specific precipitins even in the absence of a definite reduction in the leukocytes in the peripheral blood while lysins for sheep blood were little affected by it. Hektoen states that "benzene may lower the anti-infectious powers of the body in at least three ways: by the reduction of antibodies, by the reduction of the number of leukocytes, and by the reduction of the phagocytic activity of the leukocytes." This statement probably applies equally well to all three substances.

Läwen[6] found that prolonged roentgen-ray exposure of rabbits increases their susceptibility to staphylococcus infections. The resistance of mice to pyocyaneus and anthrax, and rats to anthrax infections was reduced by roentgenization. If rats and rabbits were first exposed not too long and then infected with anthrax and typhoid bacilli, respectively, there resulted a consequent increase of leukocytes as compared to animals only exposed to the roentgen rays. Roentgenization had no effect on the bactericidal action of normal serum of rabbits, guinea-pigs, and rats toward typhoid bacilli. Roentgen ray had no effect on the normal typhoid, colon and pyocyaneus agglutinins in rabbits; while the formation of specific typhoid agglutinins was distinctly depressed; it had no effect on specific typhoid agglutinins in vitro nor those contained in rabbit serum after passive transfer. There was also a marked retardation of the formation of specific typhoid bacteriolysins in roentgenized rabbits.

Regarding the effect of severe leukopenia on inflammatory reactions, Camp and Baumgarten[7] found that a congestion of blood vessels and marked edema may occur in rabbits with severe benzene leukopenia, these two phenomena being independent of the leukocytes. When the leukocyte count is below 1000, croton oil and heat produced no leukocytic exudate in the tissues of the ear, and carmine produced no exudate in the muscles. Benzene administration did not, however, destroy the leukocytes in an abscess.

The effect of leukopenia on experimental pneumococcus infections was studied especially by Winternitz and his co-workers in rabbits. They used benzene exclusively for producing leukopenia and infected their animals by intratracheal insufflation (Lamar and Meltzer). The greater part of the experiments were made with one culture of pneumococcus for the sake of uniformity. Winternitz and Hirschfelder[8] and Kline and Winternitz[9] found that pneumonia is more fatal in aplastic than in normal animals. Animals treated in like manner with toluene, a similar chemical substance causing no leukopenia, showed no decreased resistance. Animals responding to the pneumococcus infection with a leukocytosis or those having a leukocytosis produced by nutrose (sodium caseinate) seemed to have an increased resistance. In a subsequent paper Winternitz and Kline[10] find that after the intravenous injection of pneumococci in immunized rabbits the immediate reaction is not decisive of the ultimate result. The immunity process seems to be dependent on at least three factors: immune bodies, white blood cells, and a third factor which is dependent for its existence on the presence of the white blood cells at the time of the inoculation of the pneumoccoci. This third factor may be removed by rendering an immunized normal rabbit aplastic and then injecting it

[5] Ibid., 1920, 26, p. 330.
[6] Mitt. a. d. Grenzgebieten der Med. u. Chir., 1919, 19, p. 141.
[7] Jour. Exper. Med., 1915, 22, p. 174.
[8] Jour. Exper. Med., 1913, 17, p. 657.
[9] Ibid., 1913, 18, p. 50.
[10] Ibid., 1915, 21, p. 320.

with a minimal lethal dose of pneumococci. The result of the injection of this antigen into immune rabbits varies according to the presence of the three factors mentioned. The immune bodies cause an immediate disappearance of the organisms from the circulation. The third factor causes the permanent absence of the organisms from the circulation and the recovery of the animal. The white blood cells seem to be essential for the production of this third factor.

In the meantime, a reclassification of the pneumococcus into four distinct immunologic groups has been developed (Neufeld and his associates, 1910, Cole and co-workers [11]) and streptococcus hemolyticus has been divided by Avery and Cullen [12] into those from bovine sources and those from human sources, the two groups differing essentially in their ability to produce acid in dextrose mediums.

The following studies were made using the four different types of pneumococci and human and bovine strains of hemolytic streptococci. The experiments were planned to gain a general conception whether there is any difference between the effects of the roentgen ray and thorium X, both of which produce a leukopenia, on the various types of pneumococcus or streptococcus infections, and also to note whether these reagents or the leukopenia produced by them would have any effect on the invasive power or pathogenicity of the bacteria for the white mouse. Two criterions were used to determine the latter—the death of the infected and treated mice as compared to mice only infected, and the rate of appearance of the bacteria in the circulating blood.

EXPERIMENTS WITH THE ROENTGEN RAY

In all the experiments with the roentgen ray a nonlethal exposure was given the mice coincident with, or a few hours before, intraperitoneal injection. The mice, in a small box, were exposed about 6 to 12 at once for 10 minutes (Coolidge tube, the target being 8 inches from the base of the box, and using a 5 milliampere current backing up 8 inches of spark). Since it was found that the virulence of a culture of pneumococci for mice could vary in different transplants, as also noted by Wadsworth,[13] the mice were inoculated with varying amounts of the cocci in each experiment, the injections per

[11] Avery, Oswald T.; Chickering, H. T.; Cole, Rufus, and Dochez, A. R., Monographs of the Rockefeller Institute for Med. Res., 1917, 7.

[12] Jour. Exper. Med., 1919, 29, p. 215.

[13] Abst. of Bacteriol., 1920, 4, p. 20.

mouse ranging approximately from 100, 10,000, 100,000 to 100,000,000 cocci. In this way a suitable dilution was usually found that would give a consistent and distinct result in a series of mice.

TABLE 1

THE EFFECT OF ROENTGEN RAY ON PNEUMOCOCCUS AND STREPTOCOCCUS INFECTIONS IN MICE: EFFECT ON THE MORTALITY

Micro-organism		Approximate Number of Organisms Injected Intraperitoneally	Roentgenized (+) or Not (—)	Number of Mice Injected	Average Duration of Life in Hours
Name	Type				
Pneumococcus	1	100	—	4	Lived*
			+	4	88
		10,000	—	3	72
			+	4	28
		100,000	—	3	48
			+	3	30
		100 000,000	—	4	32
			+	4	22
Pneumococcus	2	100	—	3	Lived
			+	4	Lived
		10,000	—	3	78
			+	3	52
		100,000	—	4	68
			+	3	54
		100,000,000	—	3	52
			+	3	31
Pneumococcus	3	100	—	3	Lived
			+	3	Lived
		10,000	—	3	Lived
			+	4	81
		100,000	—	3	Lived
			+	3	Lived
		100,000,000	—	3	76
			+	3	52
Pneumococcus	4	10,000	—	3	Lived
			+	2	Lived
		100,000	—	3	79
			+	3	38
		100,000,000	—	2	50
			+	2	24
Streptococcus	Human	100	—	3	Lived
			+	3	60
		100,000	—	2	Lived
			+	3	61
		100,000,000	—	2	68
			+	2	42
Streptococcus	Bovine	100	—	3	Lived
			+	3	Lived
		100,000	—	2	Lived
			+	3	Lived
		100,000,000	—	2	65
			+	3	28

* The observations were made over a period of 5 days. Animals not dying before this time were recorded as living.

The summarized results of the experiments with the roentgen ray are given in tables 1 and 2 for the 6 different types of cocci.

An examination of table 1 reveals that mice subjected to a non-lethal dose of roentgen ray given shortly before infection and capable of distinctly reducing the number of peripheral leukocytes increases the pathogenicity of both pneumococci and Strept. hemolyticus, as indicated by the mortality. The pathogenicity for mice of both pneumococci and hemolytic streptococci seemed to be affected alike by the roentgen ray.

TABLE 2

THE EFFECT OF ROENTGEN RAY ON PNEUMOCOCCUS AND STREPTOCOCCUS INFECTIONS IN MICE: APPEARANCE OF COCCI IN AND DISAPPEARANCE FROM THE BLOOD

Micro-organism		Approximate Number of Organisms Injected Intraperitoneally	Roentgenized (+) or Not (—)	Number of Mice Injected	Average Time of Appearance in the Blood	Disappearance from the Blood
Name	Type					
Pneumococcus	1	100	—	3	—	—
			+	4	28′ and 34′ in 2 (2—)*	64′ and 42′ in 2
		10,000	—	3	26′ and 40′ in 2 (1—)	52′ and 50′ in 2
			+	3	Average 32′	Aver. 58′ (1 died 48′)
		100,000	—	3	Average 38′	Aver. 52′ (1 died 43′)
			+	3	Average 24′	All died 48′
		100,000,000	—	2	Average 26′	All died 50′
			+	2	Average 20′	All died 34′
Pneumococcus	2	100	—	3	46′ in 1 (2—)	46′ in 1
			+	3	36′ and 42′ in 2 (1—)	50′ and 42′ in 2
		10 000	—	3	Average 42′	Average 52′
			+	3	Average 32′	Aver. 60′ (1 died)
		100,000	—	2	Average 24′	48′ in 1 (1 died)
			+	3	Average 20′	All died 32′
Pneumococcus	3	100	—	3	—	—
			+	3	—	—
		10,000	—	2	—	—
			+	3	—	—
		100,000	—	3	32′ in 1 (2—)	40′ in 1
			+	3	Average 32′	Aver. 48′ (1 died)
Pneumococcus	4	100	—	2	—	—
			+	2	Average 30′	Average 48′
		10,000	—	2	—	—
			+	3	Average 24′	Aver. 52′ (2 died)
		100,000	—	3	Average 32′	Aver. 48′ (1 died)
			+	3	Average 22′	Aver. 48′ (2 died)
Streptococcus hemolyticus	Human	100	—	3	—	—
			+	3	32′ and 40′ (1—)	36′ and 40′
		10,000	—	3	Average 36′	Average 42′
			+	4	Average 24′	Aver. 56′ (2 died)
Streptococcus hemolyticus	Bovine	100	—	2	—	—
			+	3	—	—
		10,000	—	3	—	—
			+	4	Average 30′	Aver. 42′ (2 died)

* When all the animals in a set reveal a consistent result, the average of the total findings is given; when, however, one or two only were positive their individual results are given and those giving no findings are recorded, i. e., 2— meaning two mice gave negative findings. Likewise when an animal died this is noted.

The results in table 2 indicate that mice exposed to a nonlethal but leukotoxic dose of roentgen ray increases the susceptibility of these animals to both pneumococcus and Strep. hemolyticus infections as is indicated by the earlier appearance and longer persistence of these cocci in the blood, as compared to controls receiving equal numbers of bacteria.

TABLE 3

THE EFFECT OF THORIUM X ON PNEUMOCOCCUS AND STREPTOCOCCUS INFECTIONS IN MICE: THE EFFECT ON THE MORTALITY

Micro-organism		Approximate Number of Organisms Injected Intraperitoneally	Given Thorium X (+) or Not (—)	Number of Mice Injected	Average Duration of Life in Hours
Name	Type				
Pneumococcus	1	100	—	3	Lived
			+	4	Lived
		10,000	—	3	80
			+	3	36
		100,000,000	—	2	38
			+	3	25
Pneumococcus	2	100	—	3	Lived
			+	3	Lived
		10,000	—	3	72
			+	3	42
		100,000,000	—	2	48
			+	2	20
Pneumococcus	3	100	—	3	Lived
			+	3	Lived
		10,000	—	3	Lived
			+	4	Lived
		100,000,000	—	3	70
			+	3	36
Pneumococcus	4	100	—	3	Lived
			+	3	Lived
		10,000	—	4	Lived
			+	4	Lived
		100,000,000	—	2	64
			+	3	22
Streptococcus hemolyticus	Human	100	—	3	Lived
			+	4	Lived
		10,000	—	3	Lived
			+	3	56
		100,000,000	—	3	65
			+	2	26
Streptococcus hemolyticus	Bovine	100	—	3	Lived
			+	3	86
		10,000	—	2	Lived
			+	3	84
		100,000,000	—	3	46
			+	4	28

EXPERIMENTS WITH THORIUM X

These experiments were conducted in a manner identical with that used in the roentgen-ray experiments, with the exception that the mice received a subcutaneous injection of a nonlethal leukotoxic

amount of thorium X prepared as described in a previous communication [14] and dissolved in salt solution, in place of the ray. In preliminary tests on mice 2 units of thorium X were found to produce a distinct leukopenia from an average normal peripheral leukocyte count

TABLE 4

The Effect of Thorium X on Pneumococcus and Streptococcus Infections in Mice: the Appearance of the Cocci and Their Disappearance from the Blood

Micro-organism		Approximate Number of Organisms Injected Intraperitoneally	Given Thorium X (+) or Not (—)	Number of Mice Injected	Average Time of	
Name	Type				Appearance in the Blood	Disappearance from the Blood
Pneumococcus	1	100	—	2	—	—
			+	3	32′ in one	42′ in one
		10,000	—	3	36′ and 38′ in 2 (1—)	42′ and 46′ in 2
			+	3	Average 28′	Aver. 48′ (2 died)
		100,000,000	—	3	Average 28′	All died Aver. 52′
			+	3	Average 24′	All died Aver. 40′
Pneumococcus	2	100	—	2	—	—
			+	2	—	—
		10,000	—	3	30′ and 42′ in 2 (1—)	40′ and 42′ in 2
			+	4	Average 32′	Aver. 50′ (2 died)
		100,000,000	—	3	Average 32′	Average 46′
			+	4	Average 22′	All died 36′
Pneumococcus	3	100	—	2	—	—
			+	3	—	—
		10,000	—	2	—	—
			+	3	36′ in 1 (2—)	42′ in 1
		100,000,000	—	3	Average 38′	Aver. 46′ (1 died)
			+	4	Average 26′	Aver. 52′ (2 died)
Pneumococcus	4	100	—	3	—	—
			+	3	—	—
		10,000	—	3	—	—
			+	3	36′ and 38′ in 2 (1—)	40′ and 48′ in 2
		100,000,000	—	3	Average 30′	Aver. 52′ (2 died)
			+	4	Average 26′	Aver. 60′ (3 died)
Streptococcus hemolyticus	Human	100	—	3	—	—
			+	3	—	—
		10,000	—	2	—	—
			+	3	Average 36′	Average 40′
		100,000,000	—	3	30′ and 34′ in 2 (1—)	36′ and 40′ in 2
			+	3	Average 26′	All died 42′
Streptococcus hemolyticus	Bovine	100	—	2	—	—
			+	3	—	—
		10,000	—	2	38′ in 1 (1—)	38′ in 1
			+	3	Average 34′	Aver. 44′ (1 died)
		100,000,000	—	4	Average 28′	All died 64′
			+	4	Average 20′	All died 44′

of 13,600 per c. mm. to a minimum of 2,450 on the fifth day with complete recovery to 14,000 circulating leukocytes on the twelfth day. Five lowered the total leukocytes to about 2,100 leukocytes per c. mm. and 10 units of thorium X causing a minimum of about 1,800 leukocytes, were still nonlethal to the mature white mouse when given

[14] Corper, H. J., Amer. Rev. Tuberc., 1918, 2, p. 597.

subcutaneously. Twenty units was lethal in 7 days. The dose used in these experiments was 2 units or about $\frac{1}{10}$ of the lethal dose subcutaneously. The results of the experiments with thorium X are recorded in tables 3 and 4.

From table 3 it seems justified to conclude that thorium X shortly before infection to mice in nonlethal but leukotoxic dose capable of distinctly reducing the number of peripheral circulating leukocytes, increases the pathogenicity of pneumococci and of Strep. hemolyticus, as indicated by the mortality.

These results indicate that mice given thorium X in nonlethal but leukotoxic doses are more susceptible to both pneumococcus and Strep. hemolyticus infections, as indicated by the earlier appearance and longer persistence of these micro-organisms in the circulating blood.

SUMMARY AND CONCLUSIONS

Mice subjected to a single nonlethal exposure to the roentgen ray, capable, however, of producing a leukopenia, or given a single nonfatal injection of thorium X, also capable of causing leukopenia, and shortly thereafter inoculated with pneumococci (4 types) and hemolytic streptococci, human and bovine, revealed an increased susceptibility to all of these organisms, as is indicated by the increased and earlier mortality among the treated animals and the earlier appearance in and longer persistence of the cocci organisms in the blood, as compared with animals subjected only to inoculation.

These observations are significant since they reveal a similar increased susceptibility of the mouse subjected to these manipulations to all of the organisms tested, and bear out the results of Winternitz and his co-workers who used benzene and the pneumococcus, and Läwen, who used the roentgen ray as a leukotoxic agent and staphylococci and pyocyaneus, anthrax and typhoid bacilli as the infecting organisms. The tubercle bacillus, however, stands out distinctly from these acute micro-organisms in this respect, as noted by Corper,[2] Kellert[3] and Weinberg,[17] the course of tuberculosis in guinea-pigs being uninfluenced by the leukotoxic agents. The explanation for this difference is probably associated with the relatively greater importance of various immune processes and the defensive functions of the circulating leukocytes in the acute diseases than in chronic diseases like tuberculosis.

17 Arch. Int. Med., 1920, 25, p. 565.

THE PRIMARY TOXICITY OF CERTAIN PREPARATIONS FROM TUBERCLE BACILLI FOR MICE AND GUINEA-PIGS

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Experiments to isolate the specific immunizing or toxic substances from the tubercle bacillus have been countless. Books have been written on this subject, and still we have no concise information as to whether the tubercle bacillus produces toxic substances or whether on disintegration a toxic element is produced that acts on the animal body. Vaughan [1] believes that he has prepared the true toxic substance not only from the tubercle bacillus, but also from many bacteria and pure protein substances by disintegrating them with alcoholic sodium or potassium hydroxid. That the tubercle bacillus does not produce a true bacterial toxin, as is the case with the diphtheria and tetanus bacilli, cannot be questioned but whether it produces a substance possessing immunizing or true poisonous properties is still debatable. In 1918, Corper and Sweany [2] reported observations on tubercle bacilli indicating that these organisms, both human and bovine, possessed autolytic and a number of other hydrolytic enzymes. In the surrounding autolysate there was found to be a certain correlation between the autolysis and antigen formation. Believing that there might exist, as with the pneumococcus, a certain relation between autolysis and the formation of a toxic substance in some way related to virulence, it was decided to prepare autolysates from virulent and avirulent, human and bovine tubercle bacilli and compare their toxicities to mice, and as far as possible to guinea-pigs. Likewise it was thought that a lysis of the bacilli by means of distilled water might liberate substances which though toxic may disappear on autolysis. The later conception was strengthened by the observations by Roger,[3] in which rabbit blood laked by distilled water was toxic to rabbits, but this toxicity was absent or markedly less in autolyzed blood. It seemed desirable, therefore, to prepare distilled water extracts of

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[1] Poisonous Proteins, 1917.

[2] Jour. Bacteriol., 1918, 3, p. 129.

[3] Arch. de méd. exper. et d'anat. path., 1918, 28, p. 325.

virulent and avirulent, human and bovine tubercle bacilli, and compare these with each other and with the autolysates from the same bacilli. In the preparation of the autolysates and distilled water extracts, the tubercle bacilli[4] were obtained from glycerol broth cultures about 7 to 9 weeks old. The cultures were washed with 3 to 4 changes of sterile 0.9% sodium chloride P_H 7, Sörensen's standards, and transferred to sterile 15 c c graduated centrifuge tubes, 4 c c of bacilli in each tube, to which were then added either 10 c c of sterile 0.9% sodium chloride P_H 7 for those intended for the preparation of the autolysate, or 10 c c of sterile distilled water (P_H 7) for the preparation of the watery lysate. The autolysate was prepared by placing the bacilli, in the salt solution, in the incubator at 37 C. and keeping them there, shaking them daily, for 2 weeks when the supernatant clear fluid was obtained by high speed centrifugation. This was used for toxicity tests in mice and guinea-pigs, being preserved for use on ice. The fluids were all tested for sterility before injection. The watery lysate was prepared by placing the bacilli, in the distilled water, in the incubator at 37 C. for about 12 to 24 hours when they were placed in the icebox for the remainder of the 2 weeks, being shaken every few days. The supernatant extract was obtained by centrifugation, made isotonic by the addition of sterile sodium chloride, and was kept on ice, after testing its sterility, throughout the experiment.

The culture of avirulent human tubercle bacilli was one that was isolated in 1908 and proved avirulent for guinea-pigs, producing no demonstrable disease in doses of 1 mg. The culture of virulent human tubercle bacilli (Maxfield) was isolated from the sputum by Petroff's method in 1919 and produced generalized tuberculosis in guinea-pigs in a dose of 0.000,000,001 mg. administered subcutaneously. The culture of avirulent bovine tubercle bacilli was isolated in 1910 and produced no demonstrable disease in guinea-pigs in amount of 1 mg. given subcutaneously. The culture of virulent bovine tubercle bacilli was isolated in 1919 and produced generalized disease in the guinea-pig in a dose of 0.000,000,1 mg. subcutaneously.. Each preparation was tested by a single injection of 2 c c intraperitoneally into one mouse, and by daily injections of 1 c c intraperitoneally into 2 other mice, as stated in the table. The animals used were young mice about 4 to 5 months old.

[4] All of these tubercle bacilli were true tubercle bacilli, requiring at least 3 to 6 weeks to obtain a good growth as distinguished from certain rapidly, 2 to 4 day, growing acid fast micro-organisms which have been used in many of the investigations on this organism.

The mice were killed by means of ether and were carefully examined for macroscopic changes.

These experiments indicate that neither the autolysate nor water lysate of either human or bovine tubercle bacilli have an appreciable toxicity toward mice when given intraperitoneally in about 1 c c daily for 7 to 9 days.

TABLE 1

THE TOXICITY OF CERTAIN TUBERCLE BACILLUS PREPARATIONS, THE AUTOLYSATE AND DISTILLED WATER-LYSATE, FOR MICE

Preparation	Number of Mice Used	Intraperitoneal Injections Given in C c	Results
Human avirulent autolysate....	1	2 single....................	Negative
	2	1 daily for 9 days.........	Negative
Human avirulent water-lysate..	1	2 single....................	Negative
	2	1 daily for 4 and 10 days..	1 died from other causes
Human virulent autolysate.....	1	2 single....................	Negative
	2	1 daily for 8 and 9 days...	Negative
Human virulent water-lysate....	1	2 single....................	Negative
	2	1 daily for 8 and 9 days...	Negative
Bovine avirulent autolysate.....	1	2 single....................	Negative
	2	1 daily for 7 and 8 days...	Negative
Bovine avirulent water-lysate...	1	2 single....................	Negative
	2	1 daily for 7 days.........	Negaitve
Bovine virulent autolysate......	1	2 single....................	Negative
	2	1 daily for 4 and 8 days...	1 died from other causes
Bovine virulent water-lysate....	1	2 single....................	Negative
	2	1 daily for 7 and 8 days...	Negative

TABLE 2

THE TOXICITY OF CERTAIN TUBERCLE BACILLUS PREPARATIONS, THE AUTOLYSATE AND WATER-LYSATE FOR GUINEA-PIGS

Preparation	Number of Guinea-Pigs Used	Intraperitoneal Injection in C c	Results
Human avirulent autolysate....	2	10	Slight immediate reaction due to the amount of fluid
Human avirulent water-lysate..	1	10	Slight immediate reaction due to the amount of fluid
Human virulent autolysate.....	1	8	No reaction
Human virulent water-lysate....	1	10	No appreciable reaction
Bovine avirulent autolysate.....	1	9	No appreciable reaction
Bovine avirulent water-lysate...	2	10	Slight immediate reaction due to the amount of fluid
Bovine virulent autolysate......	1	8	No reaction
Bovine virulent water-lysate....	1	8	No appreciable reaction

Besides these tests in mice the preparations of the human and bovine tubercle bacilli were tested, so far as feasible with the material at hand, in guinea-pigs. Unfortunately, only sufficient material was available for a single large injection. Young guinea-pigs were used weighing about 300 gm. each. The results on the guinea-pigs are given in table 2.

The experiments on the guinea-pigs indicate that amounts of from 8 to 10 c c of the autolysate or water-lysate, of virulent or avirulent, human or bovine tubercle bacilli, injected intraperitoneally have no appreciable toxic action on these animals. When we consider these experiments in the light of observations by Wherry and Ervin,[5] who found that the mere grinding of lung tissue from the rabbit with 0.9% sodium chloride (0.6 gm. of lung tissue ground with 6 c c of solution) produces an extract sufficiently toxic to kill a 2 kilo rabbit when given intravenously in 0.3 c c amounts, comparatively at least, the tubercle bacillus extracts seem to be quite innocuous to animals.

Likewise 0.5 c c of an extract of the lungs of guinea-pigs given intravenously was found to be fatal to guinea-pigs. Similar extracts of liver, kidney, ileum and spleen were found relatively nontoxic to rabbits by Wherry and Ervin,

SUMMARY

The autolysate or water-lysate prepared from tubercle bacilli avirulent and virulent human and avirulent and virulent bovine bacilli in proportion of 4 parts by volume of bacilli to 10 of fluid possess no toxicity for mice when injected intraperitoneally in a single dose of 2 c c or daily for 9 days in a dose of 1 c c, nor for guinea-pigs in a single intraperitoneal injection as large as 10 c c.

[5] Jour. Infect. Dis., 1918, 23, p. 240.

WEIGHT CURVES OF TUBERCULOUS GUINEA-PIGS

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XX

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In most of the experimental work on the chemotherapy of tuberculosis, the apparently favorable results of treatment are based on the following: Increased duration of life, favorable influence on weight, and diminished distribution and progress of the disease. If it were possible in this disease in guinea-pigs to attain the ideal of all chemotherapy—complete sterilization of the animal with a single dose, it would be unnecessary to judge our success in this way. But so far no drug has been found which will completely destroy all the tubercle bacilli in a guinea-pig without also destroying the animal.

Much stress has been laid on prolongation of life as an effect of treatment. In 1917, however, it was shown by Paul Lewis [1] that the duration of life in any series of tuberculous animals is too variable to be used as an indication of therapeutic activity, unless the number of animals used is very large and the individual variations are completely accounted for. He states that with guinea-pigs, he has yet to conduct an experiment in which the last animal to die did not live at least twice as long as the first to die, and often the difference is much greater than this. No one who has worked with tuberculous guinea-pigs or with other animals having experimental tuberculosis has failed to note similar uncertainties and differences in the length of life of the animals in any given experiment, even though the animals had all received the same dose of the same strain of tubercle bacilli, the same treatment throughout and had lived under the same conditions. It is not always possible to account for these variations. Tables 1 and 2 give some of the variations in the duration of the disease in comparison with variations in weights.

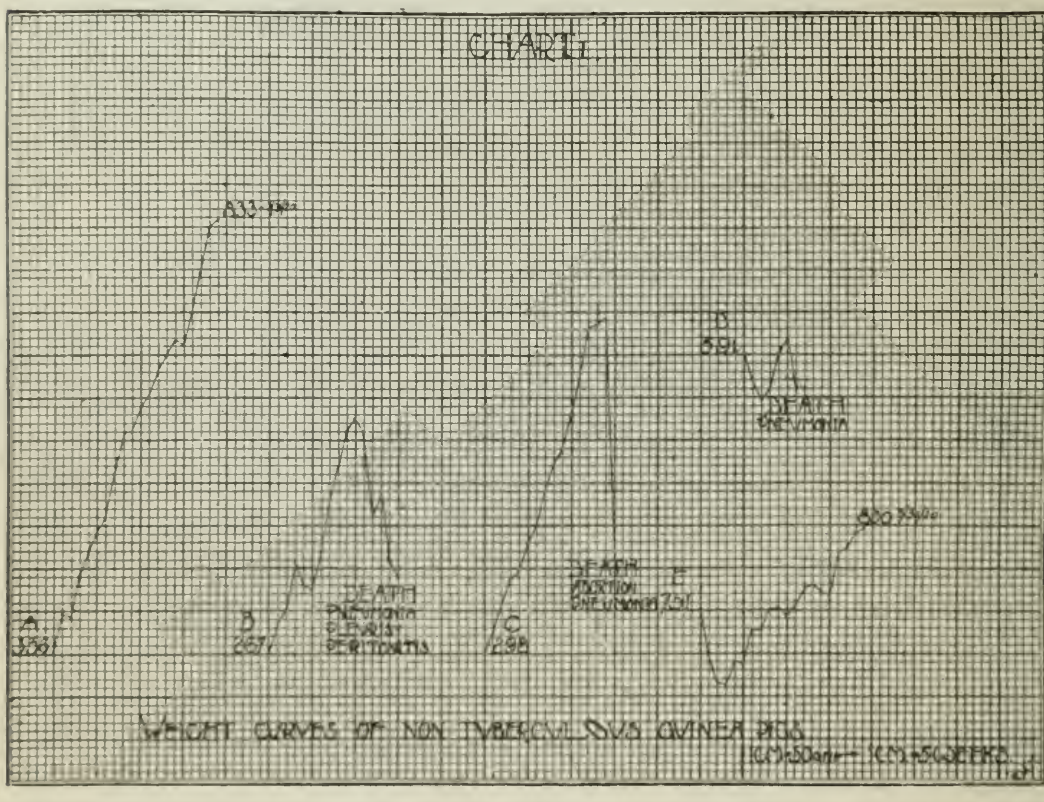
It is the purpose of this paper to endeavor to ascertain whether the weight curves of guinea-pigs inoculated with tuberculosis are any

[1] Fourteenth Report of the Henry Phipps Institute, 1918; Am. J. Med. Sc., 1917, 153, p. 625.

more uniform than the duration of life and, if not, whether the variations can be more satisfactorily explained.

For a number of years, it has been my custom to have all animals weighed once a week as a guide in treatment. Some of the earlier weights I have discarded in this paper and have used chiefly those sets the weighing of which I have personally supervised.

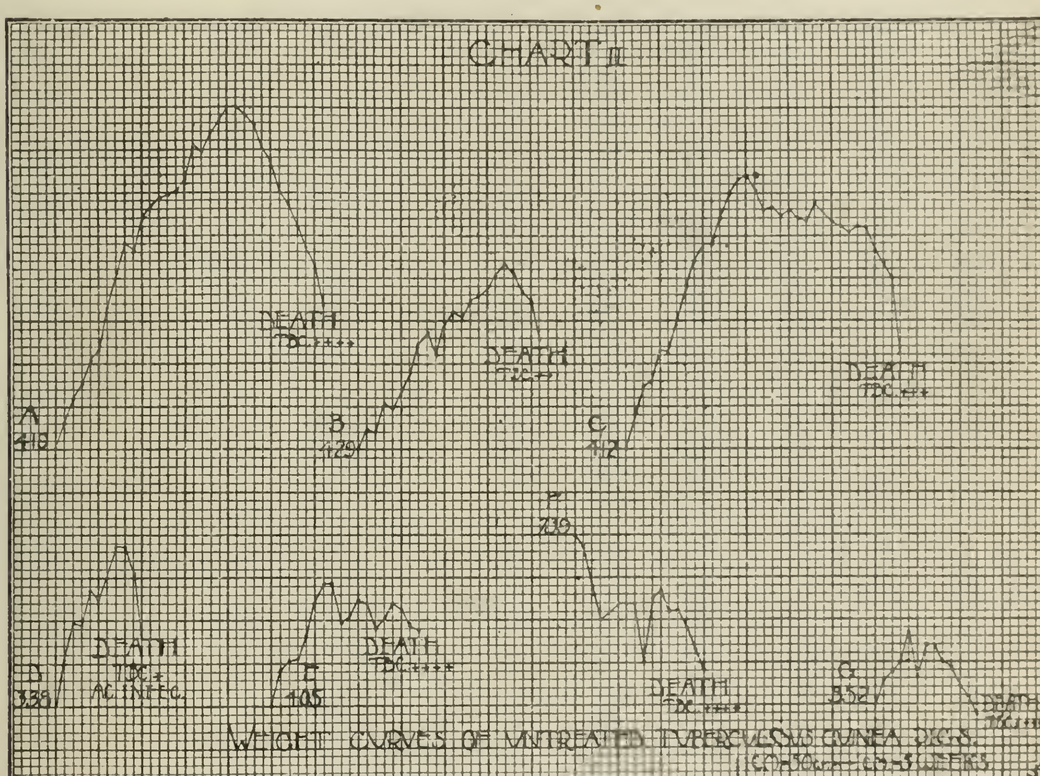
In order to have a basis of comparison, I have, during the last 22 weeks, been weighing once a week a number of nontuberculous and of supposedly normal guinea-pigs, the initial weight of which was



approximately the same as that of my inoculated animals. Chart 1 represents the type of weight curves of these animals with the cause of death in those cases in which death has occurred.

The curves in chart 1, as well as in the other charts, were made thus: The base line represents the weight of the animal at the time the experiment began—the initial weight line; this line is divided by vertical lines; one of the horizontal parallel spaces represents 10 gm. and one of the parallel vertical spaces represents one week. Nine supposedly normal guinea-pigs of different weights were used in this

experiment and were weighed once a week at the same time of the day, and, as far as possible, under the same conditions. Five of the animals died during the 22 weeks of this part of the experiment. Two died from infectious abortion with pneumonia, the weight curve (C, chart 1), showing a gradual rise as is typical of the normal growing animal and a sharp, sudden drop as seems typical of acute infections. Two, having a weight curve of the type of D, chart 1, died of an acute infection and one, having the curve B, chart 1, died from peritonitis and pneumonia. Of the 4 still living, 3 have a weight



curve typified by A chart 1, which seems to be the type of curve shown by normal, healthy, growing animals of medium initial weight. One of the heavy guinea-pigs, however, lost weight for about 3 weeks and then gradually gained and is still gaining (chart 1 E).

Chart 2 reproduces 7 of the most typical weight curves found in 37 tuberculous but untreated guinea-pigs. Table 1 itemizes the history of the individual animals used for chart 2, in which it may be noted that A, B, C and D are similar in contour, differing in height, in form of the summit, or in the width of the elevation according to

the rapidity of the ascent or descent and the length of life. An acute infection sharpens the summit and narrows the entire elevation. One of these four types is found 29 times among the 37 animals in table 1.

TABLE 1

TABLE OF WEIGHTS AND DURATION OF LIFE OF UNTREATED TUBERCULOUS GUINEA-PIGS

No. of Set	Duration of Disease, Days	Sex	Initial Weight, Gm.	Greatest Weight, Gm.	Terminal Weight, Gm.	Duration of Ascent, Weeks	Duration of Descent, Weeks	Type of Curve	Degree of Tuberculosis	Notes
1	129	F.	405	548	484	7	10	2 E	++++	
2	123	M.	386	569	408	10	6	2 A	++++	
1	131	M.	375	509	357	11	7	2 A	++++	
1	145	M.	437	589	465	10	4	2 C	++++	
1	158	M.	429	640	508	17	4	2 B	++++	
1	223	M.	419	815	589	20	11	2 A	++++	
1	78	M.	338	534	432	8	2	2 D	+	Acute infection
1	143	M.	336	605	412	8	5	2 C	++++	
1	134	M.	481	635	394	6	10	2 C	++	
1	113	M.	345	605	531	12	3	2 D	+++	
1	98	M.	360	520	436	8	6	2 B	++++	
1	246	M.	412	730	522	15	10	2 C	++++	
1	158	M.	306	568	340	15	6	2 A	++++	
1	167	M.	455	671	440	14	8	2 A	++++	
1	130	M.	430	686	457	11	5	2 D	++++	
1	116	M.	454	612	495	8	7	2 D	++++	
1	97	F.	388	533	368	5	7	1 B	+++	
1	103	M.	410	497	422	5	6	2 G	++++	
2	167	M.	352	599	432	15	9	2 A	++++	
2	169	M.	382	563	417	15	9	2 B	++++	
2	164	M.	440	674	608	15	3	2 C	++++	
2	171	M.	484	730	495	10	14	2 C	++++	
2	113	M.	375	467	326	7	9	3 J	++++	Acute infection
2	101	M.	287	441	335	9	6	2 D	++++	
3	74	F.	266	427	321	8	3	2 D	++++	
3	74	M.	264	384	292	8	3	2 D	+++	Acute infection
3	148	M.	215	401	384	12	9	2 E	+++	Killed
3	112	M.	177	344	274	14	2	2 C	++++	
3	109	M.	259	391	313	10	5	2 A	++++	
4	27	F.	590	680	660	2	1	2 D	++	Acute infection
4	60	F.	240	290	205	4	2	3 B	++++	
4	104	M.	295	425	255	10	3	3 C	++++	
4	105	M.	580	685	610	7	3	2 E	++++	
4	105	F.	730	730	500	0	15	2 F	++++	
5	82	M.	352	438	341	4	6	2 G	+++	
5	76	M.	352	395	309	5	5	2 G	++++	
5	69	M.	378	410	310	2	6	3 A	++++	

Set 1 of table 1 consists of 18 guinea-pigs, varying in weight between 350 and 450 gm., which were inoculated at the same time with the same amount of the same strain of human tubercle bacilli. Six received the ordinary laboratory feeding of carrots, oats and hay. Twelve received lettuce and specially prepared graham crackers and hay. Hence the animals of set 1 differed only in their food. The weight curves of 16 of the 18 can be classed under the 4 types which I regard as typical for tuberculosis in guinea-pigs. One had the weight curve E, chart 2, which differs from C only in the fact that

death occurred very near the summit of the elevation and that the ascent was not so high. One had the curve G which differs from B in being lower and in having a double apex. Set 2, table 1, is composed of the 6 untreated controls of our cresol experiments. They were inoculated with the same strain of human tubercle bacilli as was set 1, but received four times as large a dose. Five of these had curves of one of the four main types, but one, which developed an acute peritonitis in addition to the tuberculosis, showed a curve similar to J, chart 3, which is formed much like the typical curve, but is lower and descends below the base line.

Set 3 consisted of 5 guinea-pigs inoculated with the washings from garnets used as controls in a bactericidal experiment. The garnets were covered with an ùnkonwn quantity of tubercle bacillus suspension, then exposed to salt solution, washed and shaken in salt solution. Hence the dose used in the inoculation was unknown. However, of the 5 animals whose curves were taken, 4 had the typical curves A, C and D, while one had curve E, in that it was killed near the apex of the curve. Set 4 was inoculated with an unusually large dose of a very virulent strain. As a result, the duration of the disease was relatively short and the curves less typical than in the other sets. Set 5 was also a bactericidal control. Only three curves were used, since most of the animals are still living. Curve G reproduces the curve in two of these. The sudden drop shown in this curve was due to the fact that we had had no carrots for 4 days, so that during this time they had lived on oats and hay. The same explanation applies to curve A, chart 3, which more nearly resembles the curve of the third animal. In studying table 1 and chart 2 in the effort to determine the causes of the typical curves and of any modifications from the types, we note that most of the animals were young adults, still growing but past some of the weaknesses which belong to the young. Extreme age, as indicated by 2F and 1E, tends to modify the curve considerably. The natural tendency of the weight curve in the normal growing animal is constantly upward, as shown in A, chart 1, and since the inoculation with a small or moderate dose of a moderately virulent strain of human tubercle bacilli does not materially influence health at first, the curve follows its natural upward tendency until something in the way of diet or secondary infection or an overpowering of the animal with the effects of the tubercle bacillus impairs the health and the curve begins to descend. Larger initial doses or a more

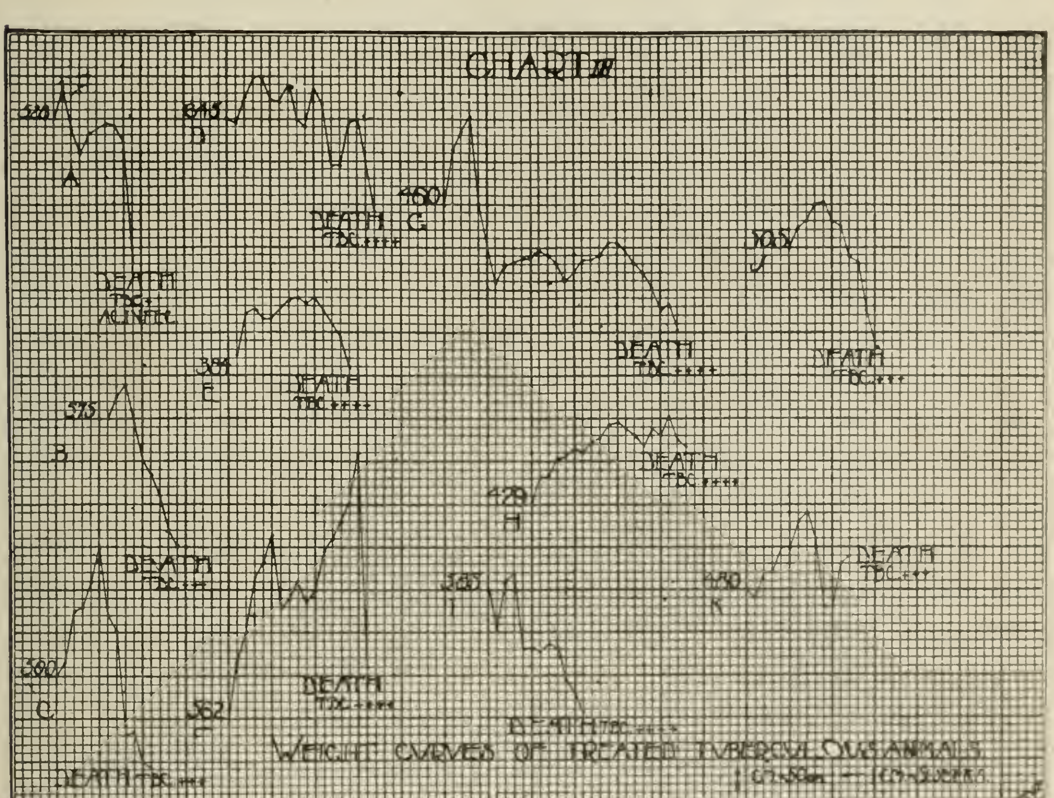
virulent strains of organisms tend to cause shorter ascending curves and thus a lower elevation. Secondary infections may cause sharp, sudden descent. Sex seems to have some effect on the shape of the curve, since of the 6 females in table 1, 4 had more or less atypical curves, while only 8 of the 30 males had curves varying in any way from the typical.

It is not always possible to explain variations. It is even difficult to explain the typical curve. The ascent is easy to explain since that is natural. But why the occasional sharp breaks in the ascent? Why the final slow or rapid descent? Why the sharp or rounded apex in some cases and the broad but jagged plateau in others. We have already seen how variations in the diet may cause decided drops in the weight and these or slight infections may explain the occasional jagged outline of the ascent. The final descent does not necessarily mean the generalization of the tuberculosis, since several animals that died or were killed at or near the apex of the curve showed all the internal organs involved in the tuberculous process. We must, therefore, assume that the descent is due to the overpowering of the animal by the toxic products of the bacteria. Whether the apex shall be broad or narrow probably depends on whether the resistance gives way suddenly or gradually—in other words, on the individual factor in the equation.

The question of the effect of treatment on the weight curve can be answered only so far as it concerns the 56 cases whose weight curves have been plotted. No generalization can be made from this number since it is self-evident that the effect of treatment on the weight curve as well as its effect on the condition of the animal, on the progress of the disease and on the length of life must vary with the treatment. Each drug used will probably affect the curve differently.

Chart 3 gives in graphic form some of the types of curves found in the 56 animals which had received six different modes of treatment. The curves were plotted as in charts 1 and 2. Table 2 gives in detail the individual animals of these various sets, with the treatment of each, the duration of life, the weights and the type of weight curve shown, the numeral referring to the chart and the letter to the curve. Many of the curves varied so little from those of the untreated animals that the type in chart 2 or 1 can be used in describing these treated animals. Chart 3 therefore reproduces in the main those types

that are quite different from those curves in the untreated animals, i. e., atypical curves. As may be seen from table 2, four of the 6 guinea-pigs treated with orthocresol had typical tuberculosis curves and only 2 the less typical curves F and G of chart 3. Metacresol and paracresol treatments, on the other hand, give only 2 characteristic curves from chart 2, and 4 curves from chart 3. Two of the guinea-pigs treated with thymol, which died of an acute infection and had little tuberculous involvement had weight curves similar to I and J of chart 3, three had typical weight curves and one, which is still living, has so far the typical



weight curve of a normal animal, as represented in A of chart 1. Of 6 animals treated with mercurophen, 3 had typical curves of untreated animals, while 2 had a low elevation shaped like the typical curve and 1 had a triple crested curve like E of chart 2. Of the 11 animals treated with the double salt of mercuric chlorid and methylene blue and the 8 treated with the same double salt combined with a vaccine of human tubercle bacilli, only 1 was typical, all the others showing some one of the abnormal types in chart 3. Five tuberculous guinea-pigs treated with the double salt of mercuric chlorid and methylene blue are still

TABLE 2

Table of Weights and Duration of Life of Treated Tuberculous Guinea-Pigs

Duration of Disease, Days	Sex	Initial Weight, Gm.	Greatest Weight, Gm.	Terminal Weight, Gm.	Duration of Ascent, Weeks	Duration of Descent, Weeks	Type of Curve	Degree of Tuberculosis	Treatment
113	F.	562	879	580	10	3	3 F	++++	Orthocresol
188	F.	460	562	314	13	12	3 G	++++	Orthocresol
113	M.	553	664	475	8	9	2 B	++++	Orthocresol
87	F.	427	613	401	9	4	2 D	++++	Orthocresol
142	F.	586	822	754	19	2	2 B	++++	Orthocresol
118	M.	324	480	387	8	9	2 A	++++	Orthocresol
98	M.	355	551	434	8	5	2 A	++ *	Metacresol
74	F.	575	654	436	7	4	3 J	++++	Metacresol
87	M.	362	535	425	9	4	2 B	++ *	Metacresol
107	M.	415	513	440	5	3	3 E	+++	Metacresol
80	F.	585	611	425	2	6	3 I	++++	Metacresol
73	M.	503	554	357	4	6	3 J	+++	Metacresol
170	M.	415	556	440	14	8	2 C	+++	Paracresol
120	M.	389	491	373	10	4	3 E	+++	Paracresol
134	M.	429	534	499	16	2	3 H	+++	Paracresol
162	M.	390	534	400	12	5	3 E	+++	Paracresol
134	M.	344	561	457	17	2	2 B	++ *	Paracresol
58	M.	455	556	402	3	5	3 J	+ *	Thymol
80	M.	483	554	386	6	6	3 I	++ *	Thymol
115	M.	396	547	419	10	6	2 C	++ *	Thymol
136	M.	390	553	394	8	12	2 B	++++	Thymol
147	M.	357	486	337	9	11	2 A	+++	Thymol
	M.	413	...	...	..	..	1 A	......	Thymol
89	M.	420	490	442	6	4	2 E	++ *	Mercurophen
139	M.	368	650	617	16	3	2 C	++++	Mercurophen
71	M.	367	501	387	8	2	2 D	+ *	Mercurophen
107	M.	430	551	493	7	1	2 C	+ *	Mercurophen
97	M.	384	465	383	10	4	3 E	+++	Mercurophen
132	M.	428	549	397	12	6	3 E	++++	Mercurophen
120	F.	380	515	435	11	6	3 F	+++	$HgCl_2$ methylene blue
114	M.	510	665	505	12	5	2 B	++ *	$HgCl_2$ methylene blue
68	F.	565	610	390	1	6	3 B	+++	$HgCl_2$ methylene blue
87	F.	500	650	385	5	6	3 C	++++	$HgCl_2$ methylene blue
75	F.	575	615	425	3	7	3 B	+++	$HgCl_2$ methylene blue
68	F.	480	570	520	9	3	3 K	+++	$HgCl_2$ methylene blue
76	M.	480	515	335	5	5	3 B	+++	$HgCl_2$ methylene blue
56	F.	440	455	330	2	5	3 I	+++	$HgCl_2$ methylene blue
75	F.	585	710	510	4	4	3 G	+++	$HgCl_2$ methylene blue
62	M.	528	565	358	4	6	3 A	+ *	$HgCl_2$ methylene blue
73	M.	572	572	364	3	6	2 F	+ *	$HgCl_2$ methylene blue
142	M.	450	550	445	8	8	3 E	++++	Tuberculosis vaccine and $HgCl_2$ methylene blue
129	M.	695	765	545	6	10	3 F	++++	Tuberculosis vaccine and $HgCl_2$ methylene blue
156	F.	620	665	530	5	5	3 G	++	Tuberculosis vaccine and $HgCl_2$ methylene blue
143	M.	645	695	535	6	9	3 D	+++	Tuberculosis vaccine and $HgCl_2$ methylene blue
153	M.	690	725	650	7	6	3 A	+++	Tuberculosis vaccine and $HgCl_2$ methylene blue
85	F.	520	540	410	2	8	3 B	++++	Tuberculosis vaccine and $HgCl_2$ methylene blue
66	M.	685	710	555	3	4	3 B	—	Tuberculosis vaccine and $HgCl_2$ methylene blue
110	F.	445	525	495	12	3	2 E	++++	Tuberculosis vaccine and $HgCl_2$ methylene blue
60	F.	575	575	325	0	10	2 F	++++	Iodine in starch
100	M.	675	685	500	4	7	3 J	++++	Iodine in starch
78	M.	590	620	510	4	5	3 J	++++	Iodine in starch
77	F.	625	625	470	3	6	2 F	++++	Iodine in starch
90	M.	415	535	335	7	6	2 E	++++	Iodine in starch
95	M.	470	530	395	5	8	2 G	++++	Iodine in starch
138	M.	409	469	334	9	9	3 E	++++	Iodine in milk
117	M.	341	462	321	8	8	3 E	++++	Iodine in milk

* Acute infection

living, but their weight curves up to the present time do not conform to the types that we have regarded as typical for untreated tuberculous animals. Two sets of animals have been treated with iodin. In the first set, the iodin was given in powdered starch and in these the curves were all more or less atypical. Of the second set, which have been fed iodin in milk powder, only two have died, showing the low but typically shaped curve represented in E of chart 3. Five of the guinea-pigs of the second set are still living and so far their curves correspond with the normal curve A in chart 1 or with the beginning of A or C of chart 2.

In order to determine how much of the effect on the weight curve in the treated animals was due to the treatment and how much to the treatment combined with the tuberculous infection, 3 normal guinea-pigs have received the same treatment as each set of pigs given in table 2 with the exception of the iodin in powdered starch and the tubercle bacillus vaccine combined with the double salt of mercuric chlorid and methylene blue. Of the 21 normal guinea-pigs thus treated whose weight curves have been thus charted, 4 have died of acute infections and have the curve of most acute infections. The rest are living after 3 and 5 months' treatment and their weight curves correspond with A of chart 1. In other words, if uninfected, they run, in spite of the treatment, the typical weight curve of the normal animal; that is to say, while these drugs modify considerably the weight curves of tuberculous animals, they have not, during the months of the experiment, changed the weight curves of normal, uninfected guinea-pigs. In the 37 untreated tuberculous animals, the height of the elevation, i. e., the difference between the initial weight and the maximum weight averaged 166 gm. and, if sets 4 and 5 of table 1 be omitted because of the larger dosage and greater virulence of the infectious organisms, the average height of the elevation is 195 gm. In the 55 treated tuberculous guinea-pigs, on the other hand, the average height of the elevation was only 100 gm. If, however, we wish to compare the average elevations of the tuberculous animals under the different methods of treatment, we find the average weight elevation of the 22 animals treated with the cresols and thymol is 139 gm.; the 6 tuberculous animals treated with mercurophen have an average weight elevation of 135 gm. The tuberculous animals treated with the double salt of mercuric chlorid and methylene blue have an average weight elevation of only 75 gm., while that of the 8 animals

treated with the same double salt and a vaccine of killed tubercle bacilli is 53 gm. Fifty-four grams is the average weight elevation of the 8 iodin-treated pigs.

If we compare the treated animals with the untreated with reference to the descent of the weight curve below the line of the initial weight, we find that only 11 of the 37 untreated animals had curves descending below the base line, while 37 of the 55 treated animals had such long descending curves. The average distance of the terminal weight below the initial weight in the untreated animals was 56 gm., while only 1 animal had a curve descending more than 100 gm. below the base line. The treated animals, however, showed an average distance below the base line of 95 gm., while 18 of the 37 had curves descending more than 100 gm. below the base line.

CONCLUSIONS

Normal guinea-pigs of approximately the same age and weight and living under the same conditions run a uniform weight curve. This curve is easily modified by changes in diet, by acute infections and other variations in the conditions of life.

Normal male guinea-pigs of approximately the same age and weight inoculated with the same dose of the same strain of tubercle bacilli and living under the same conditions, run a fairly uniform and typical weight curve. This weight curve may therefore be used in testing the effect of various methods of treatment and is a more reliable standard than the duration of life.

Most chemotherapy, so far as tested, even though the drugs and doses used are so nontoxic as not to interfere materially with the duration of life or with the weight curves of normal, uninfected guinea-pigs, tends to alter materially the type of weight curve. This alteration consists in the main in a diminution in height of the ascending curve and an increase in length of the descending curve.

It may be inferred that the more closely the weight curves of tuberculous animals treated by any method adhere to the normal weight curve, the more benefit we may hope for from the treatment.

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HEMORRHAGIC MENINGOENCEPHALITIS IN ANTHRAX

A REPORT OF THREE CASES

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From the Pathological Laboratory of Rush Medical College, Chicago

Although there are reasons to believe that localization of the bacilli in the brain is relatively common [1] in anthrax the cerebral changes produced are, so far as I am able to find, as yet undescribed in American literature. As there is at present an increasing incidence of anthrax in this country, a report at this time I believe is appropriate, especially in so far as it may assist in the recognition of such infections either clinically or at the necropsy table.

Three of the deaths from anthrax in Chicago within the past 6 months have yielded the brains on which this report is based and in only one of these was anthrax suspected before death. These 3 deaths were subject to medicolegal inquiry.

Case 1.—The first case was that of a white boy, aged 15 years, who was admitted, in coma, to the Cook County Hospital, service of Dr. Friedberg. From the father a history was obtained of onset two days before with headache and sore throat and also the history of an old ear trouble. On admission the temperature was 99.4 F., pulse 120, respirations 26; physical examination revealed an acutely ill young man in coma with a swelling of the left side of the neck, hyperemia and edema of the vocal cords, and a leukocyte count of 75,000 (97% polymorphonuclears). He lived 4 hours in the hospital and died 65 hours after the onset. A clinical diagnosis was not made, but mastoid abscess with lateral sinus thrombosis was suspected.

Anatomic diagnosis (Dr. E. R LeCount): Acute hemorrhagic leptomeningoencephalitis (anthrax infection); anthrax of the left side of the neck, scalp and chest; hyperemic papules of the face (anthrax?); acute anemia.

The skull cap came away with ease. The dura was everywhere tense and was light purple from the blood underneath. In the superior longitudinal sinus there was fluid blood only. The brain was everywhere on top bright red from the blood in the pia arachnoid meshwork. There was a little more blood in the front than in the back half; there was enough in the front half to hide entirely the convolutions, while in the back half there was only a thin layer of blood on the summits of the convolutions, the sulci here being filled. There was a small amount of viscid blood stained material between the two layers of the arachnoid but no free blood. Where the pia arachnoid was stripped from the surface of the convolutions numerous red pinpoint spots remained, as large as 1 mm. in diameter, which stippled these surfaces quite uniformly. The brain substance was so swollen that the convolutions were

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[1] Lancet, 1880, 1, pp. 871 and 909; 1901, 2, p. 133; 1905, 2, p. 934.

almost flat on their tops, and the sulci almost closed. The brain, especially the gray matter, was soft. The large arteries of the base of the brain were unchanged. The cerebrospinal fluid was blood stained. The brain was put in formalin solution and the examination continued three days later.

On the two broad surfaces made by one horizontal cut through the corona radiata there were hundreds of 1 to 2 mm. petechial hemorrhages, mostly oblong and irregular, limited practically to the cortex, and as a rule perpendicular to the outside surface of the brain (fig. 1,a). They were about the same distance apart and had a tendency to widen out in the deeper layers of the cortex (fig. 1,b). There were few such hemorrhages in the white matter, but the small vessels here were greatly distended with blood. A similar section was made through the basal ganglions (fig. 1), and in addition to the characteristically distributed cortical hemorrhages there were numerous hemorrhages in the lenticular and caudate nuclei, both thalami, and the hippocampi. These hemorrhages were not confluent as they may seem in the photograph, but were separated by narrow bands of dark gray brain substance. The largest of these places of hemorrhage had a surface measurement of 1.5 by 5 mm. The hemorrhages of the right thalamus and head of the right caudate nucleus were so numerous as to cause these structures to bulge into and narrow the adjacent ventricles (fig. 1). There were three or four rather large (1 to 3 mm.) hemorrhages of the gray matter of the left dentate nucleus of the cerebellum.

Bacteriology.—Anthrax was not suspected until after death. Cultures were made from the brain substance, superior longitudinal sinus blood, and cerebrospinal fluid and from each a gram-positive rod shaped bacillus grew in pure culture on human blood-agar plates. This bacillus answered all of the cultural and morphologic requirements for anthrax and killed guinea-pigs inoculated subcutaneously in 12 to 18 hours, producing anthrax symptoms, large spleens, and anthracemia (encapsulated bacilli if examined before the death of the pig). The bacillus was also grown in pure culture from the fluids and tissues of these pigs. Meningeal hemorrhage was not found in either of the two pigs examined, but it is known to occur from such experiments (Gilmour and Campbell[2]).

Microscopic Examination.—A perfect series of sections was made from the cerebral cortex at a place chosen at random and cut tangent to the outside surface, beginning at the visceral layer of the arachnoid, extending through the cortex and into the white matter for 6 to 7 mm. Sections not in series were taken from other places and corresponded in all details to those of the series.

The visceral layer of the arachnoid was in places two or three times its normal thickness due to the separation of its delicate connective tissue fibers by a cellular, slightly fibrinous and bacillus laden exudate, similar to that in the pia arachnoid meshwork. Within the meshes of the pia arachnoid there was a large amount of exudate and great numbers of bacilli, in the spaces mostly red blood cells. This exudate was for the most part composed of red blood cells and leukocytes (large mononuclear and polymorphonuclear). There were also a few endothelial and plasma cells, large vacuolated mononuclear cells, and a small amount of fibrin. Intermixed with this there were enormous numbers of bacilli. Nowhere was the exudate entirely free from red blood cells, but the latter were most numerous in the dilated spaces. There was very little disintegration of the leukocytes of the exudate, a few of which, however, contained whole or parts of broken bacilli.

[2] Canadian Med. Assn. Jour., 1918, 8, p. 97.

Fig. 1.—Horizontal section (superior surface) through the basal ganglions of the brain (case 1). The hemorrhages of the cortex are of rather uniform size, many of them perpendicular to the pia (a), and many widened out in the deep layers of the cortex (b); no region of the cortex is spared; very few hemorrhages of the white matter; the hemorrhages in the lenticular and caudate nuclei are small, not confluent, but separated by dark gray strands of brain tissue. Note the predominance of hemorrhages (c) along the course of Charcot's artery in the outer portion of each lenticular nucleus.

The chief changes in the leptomeninges were those of the vessel walls. The adventitia of practically every vessel (artery and vein) was laden with bacilli and leukocytes. The walls of these vessels were thicker than normal, the individual elements being spread wide apart by edema. As a result the walls were weakened and it was common to find them ruptured. The intimal changes were least marked and consisted of patchy proliferation of the endothelium and subendothelial infiltration with leukocytes (fig. 6). It was on such places as this that thrombi were found. There was an increased number of leukocytes in the lumens of all the cerebral vessels.

One of the early changes of the wall was a partial or total separation of the adventitia from the media that may be present with a still intact media or after the rupture of the media with a still intact intima, in the last instance forming what is described as "aneurysma desiccans." In a few arteries the wall was greatly infiltrated with red blood cells and leukocytes, the intima being still intact, bridging over the defect. This was not so common in the veins.

The delicate fibers of the pia mater were moderately separated by exudate and bacilli. Bacilli were also numerous in the adventitia sheaths of the arteries and veins as they entered the cortex and could be traced in this situation through the entire gray matter and into the underlying white substance. Mixed with these bacilli was a hemorrhagic leukocytic exudate which could be traced back to that of the pia mater (as in fig. 2 b,c). Between the pia and the surface of the brain substance there was, in places, a layer of finely granular coagulated plasma as thick as 300 mikrons, with an occasional nonstaining cell. In this layer of plasma and below it there were occasional bacilli. Between the pia and the brain there were also, in places, numerous red blood cells which were often in direct continuity with similar cells in the perivascular spaces of the brain substance (fig. 2 d, d′).

In the gray matter of the cerebrum the essential changes were vascular. Hardly a single arteriole or venule was unchanged. There were bacilli in the adventitia of these vessels. In the perivascular space surrounding practically every vessel there were red blood cells, so numerous in some as to distend them to a diameter 12 or 15 times greater than the vessel. If such a vessel were followed along in the serial sections, one or more places of rupture might be found in its wall. From these dilated perivascular spaces the red blood cells extended out in irregular finger-like masses, forming the petechial hemorrhages seen grossly. In these hemorrhages, as well as in the perivascular spaces, there were only a few scattered bacilli except in the neighborhood (100 to 200 mikrons) of the breaks in the walls of the vessels. Here the bacilli, present only in the adventitial spaces at other levels, occurred in relatively small numbers, both in the perivascular spaces, in the places of hemorrhage, and even in the surrounding brain substance. The venules of the gray matter were altered to a slightly greater degree than the arterioles.

As the white matter of the cerebrum was approached in a study of the serial sections, all these changes became progressively less. The changes of edema of the interstitial brain substance, the granular changes in the protoplasm of the ganglion cells, became less. The loss of chromatin and the poor staining qualities (small darkly stained nucleus or faintly staining nucleus) of the nuclei of the ganglion cells was less marked in the deeper parts of the gray matter. In the white matter the lumens of the vessels were greatly distended with red blood cells. In no section were bacilli found inside the vessel lumens, either of the brain or its meninges.

CASE 2.—A. K., a laborer, aged 62 years, entered Cook Coutny Hospital, the service of Dr. Hollinger, in coma, suffering from air hunger and cyanosis and with an indefinite history of an onset three days previously. He was a laborer in a "curled hair" establishment handling hair from South America.

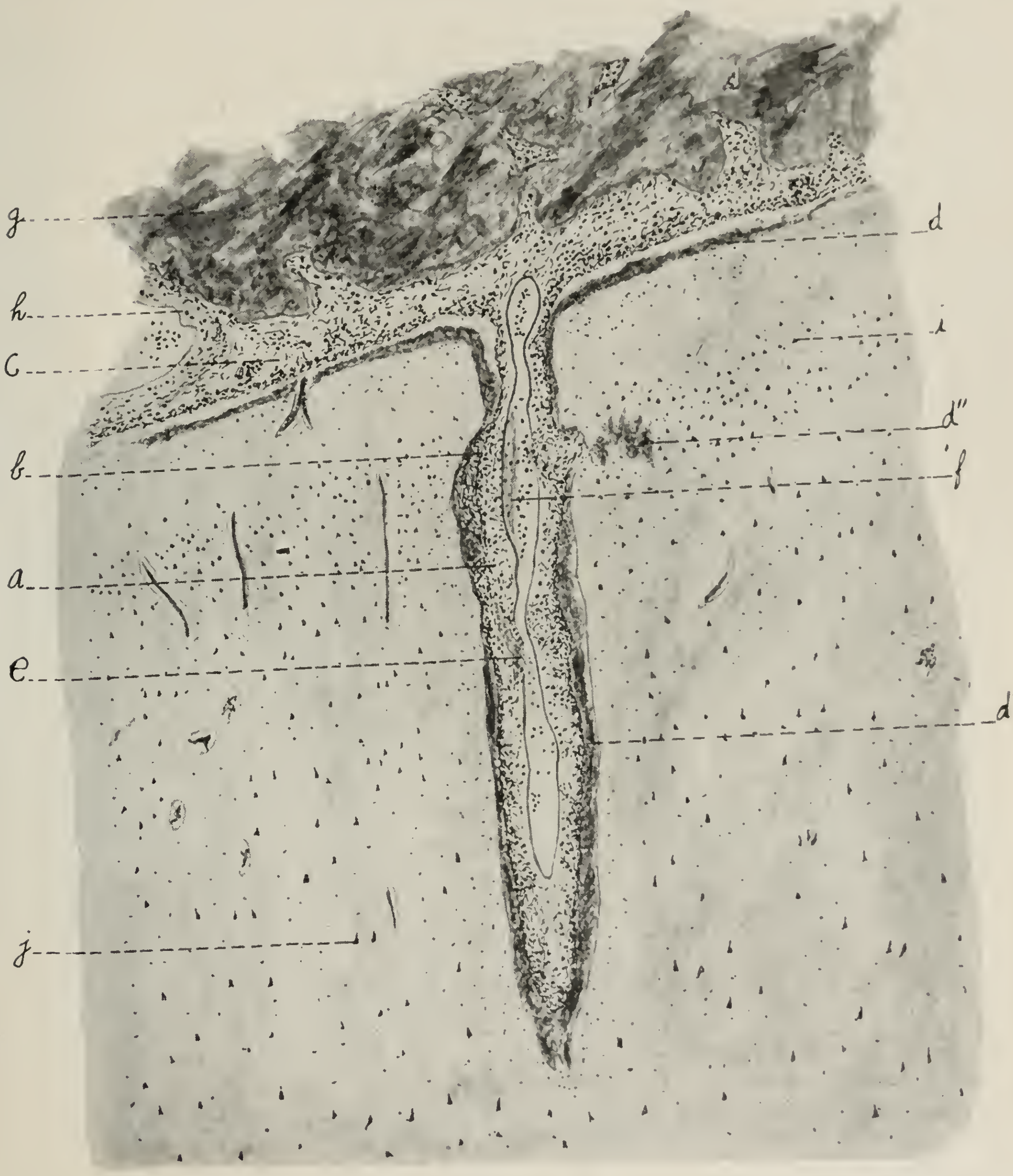

Fig. 2.—Longitudinal section of an arteriole entering horizontally the substance of the left middle frontal gyrus from the superior frontal sulcus (case 2). (A similar vessel is shown cut cross-wise in fig. 3.) The fibers of the adventitia (a) are dissected widely apart by edema and in this layer of the vessel there are numerous leukocytes mixed with bacilli (b) which are continuous with similar elements in the pia mater (c); also the blood underneath the pia (d) is continuous with that outside the adventitia of the vessel (d'); the intima is broken through at (e) and a mural thrombus is indicated by (f), blood in the pia arachnoid spaces by (g), leptomeningeal artery (h), outer layer of ganglion cells (i), large ganglion cells (j); the mass of red blood cells (d") is continuous with that outside the adventitia (d'); Gram-Weigert stain; camera lucida.

On admission his temperature was 104.4 F., pulse 120, respirations 36. There was a marked soft swelling behind the right tonsil and of the soft palate, also an indurated swelling of the right side of the neck with a small pustule opening at the summit of the swelling. This swelling continued onto the right side of the chest. The pustule had a small necrotic center with a blue margin and a red zone about this. Bacilli were found in a smear from this pustule. The extremities were spastic. The pustule was excised and tracheotomy made. There were numerous small lacerations and bruises of the scalp and extremities from having fallen out of bed at home (convulsions?). The leukocyte count was 28,000 and the cerebrospinal fluid blood stained. He lived about 10 hours in the hospital, and died about 3 days after the onset. A diagnosis of anthrax was made.

Anatomic diagnosis (Dr. E. H. Hatton): Acute hemorrhagic leptomeningo-encephalitis (anthrax infection); anthrax of the right side of the neck; edema of the glottis; acute hemorrhagic cervical lymphadenitis; acute anemia; acute hyperplasia (220 g.) of the spleen; recent tracheotomy; multiple small lacerations and bruises.

Brain.—The dura is everywhere tense and pale blue from the blood underneath it. When the dura is reflected, blood within the leptomeninges comes into view. This blood is thickest (2-6 mm.) at the frontal and parietal lobes. The convolutions of the top and base of the brain were only slightly visible shining through as gray opaque places. Wherever the tense arachnoid was torn in handling the brain, the surfaces of the convolutions were studded with petechial hemorrhages, 0.5 to 1 mm. in diameter, rather evenly distributed. On the cut surface of the cortex these hemorrhages were irregular, oblong, 1 to 2 mm. in diameter and limited almost entirely to the gray matter. The brain was extremely soft, almost jelly-like and weighed 1,470 gm. The cerebrospinal fluid was blood stained. The large arteries of the base of the brain were unchanged. A horizontal section through the basal ganglions made surfaces essentially similar to that shown in fig. 1. The hemorrhages were a little more numerous in the opercular and insular regions than elsewhere in the cortex but otherwise the distribution, size, shape and position (relative to the gray matter) were similar to brain 1; the white matter was similarly spared. The hemorrhages of the basal ganglions, though not quite so numerous as in brain 1, were similarly distributed. These hemorrhages were a little more numerous along the outer borders of the lenticular nuclei and along the vessels crossing the anterior limbs of the internal capsule. Such an arrangement was also present in brain 1.

Bacteriology.—In the stained smears of cerebrospinal, right and left pleural, and pericardial fluids and heart blood, large long-chain, square-end gram-positive bacilli were seen and pure cultures of similar bacilli were grown from each of these fluids. These were identified as anthrax, and pure cultures inoculated into guinea-pigs caused death within 20 hours. Hemolytic streptococci were also found in the pleural and pericardial fluids.

Microscopic Examination.—The changes in this brain differ from those in case 1 only in degree. There was more of a similar exudate in the pia mater, distending it to many times its normal width. The cerebral arterioles and venules had wide adventitia sheaths, the fibers of which were spread apart by a leukocytic bacillus-laden exudate and granular débris, stretching the wall to many times its normal width, the lumen either remaining normal with an intact intima or narrowed slightly by mural thrombi (fig. 3,e). When one of these vessels was followed along, an occasional break was found in its wall. At

such a break there were red cells at the periphery of the already overdistended perivascular space and from here finger-like masses of red blood cells extended out into the brain substance (fig. 3c). There was continuity between the exudate in the pia and that in the adventitia sheaths of the cortical arteries and veins (fig. 2 a,b,c). Many of the adventitia sheaths were so widened that the surrounding brain substance was compressed, forming round or oval lightly-staining places, easily visible to the unaided eye when the stained section was held up to the light and as large as 0.5 mm. in diameter, when the normal diameter of the vessel would be about 0.02 mm. This increase in diameter was due primarily to changes in the adventitia. The few bacilli in the brain substance were either in the places of hemorrhage or in the bud-like extensions outward of the adventitia of some vessel (fig. 3a), in every case a continuity

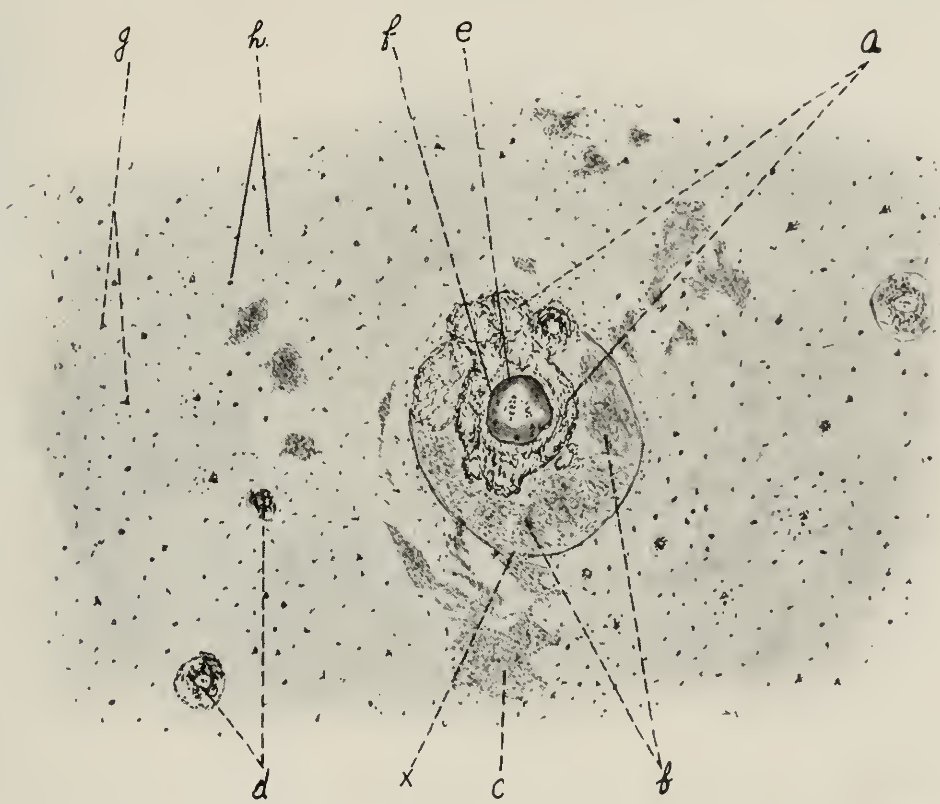

Fig. 3.—The cross section of an arteriole in the cortex of the left middle frontal gyrus 1 mm. in from the surface of the brain (case 2). The adventitia (a) is dissected widely from the media by edema and between it and the media there are many bacilli. There is a great deal of blood (b) outside the adventitia and this is continuous (x) with other masses of blood (c) still further out in the brain substance. In fig. 2 this blood about the vessel is but a part of the hemorrhage of the pia arachnoid and underneath the pia. This location of bacilli between the adventitia and media is also present in smaller vessels (d); a mural thrombus is indicated by (e), intima by (f), ganglion cells (g), glia cells (h). Gram-Weigert stain.

being easily established with some vessel. Only 3 bacilli were found in the vessel lumens during the careful study of several hundred sections. In no section were changes found in the intima except when associated with marked changes in the adventitia and media. Leukocytes were numerous in all these cerebral vessel lumens. The changes, other than already mentioned of the brain substance, were those of edema and acute inflammation, the increase in

glia cells, satellitosis and shrinkage of the ganglion cells being most marked. All these changes became progressively less the deeper the section was taken.

Case 3.—L. K., a white man, aged 36 years, single, a laborer in a "curled hair" factory handling hair from South America, complained for the first time on the evening of May 1, 1920, of pain in the left side of the neck and left shoulder. May 2 (Sunday) he did not complain. May 3 he did not go to work; he complained some but helped his landlady about the housework. This history was obtained through an interpreter from the landlady, who could speak no English and appeared not to be very intelligent. On May 4 he drank some coffee and at 8:30 went to the toilet. He came out suffering from extreme dyspnea and collapsed in a chair. The dyspnea increased, he became more cyanotic and died at 9 a. m.; there was no attending physician and no diagnosis had been made.

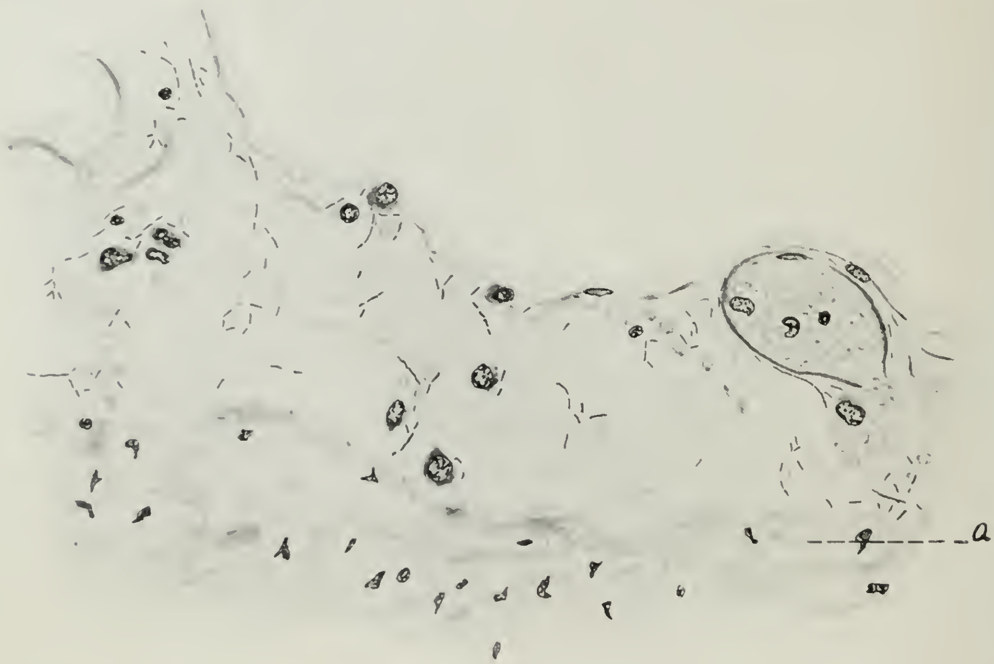

Fig. 4.—The pia with its fibers spread widely apart by edema (case 3). There are a few leukocytes and many bacilli in it; the cortex (a) underneath; gram-Weigert stain; Camera lucida.

Anatomic Diagnosis (Dr. J. P. Simonds).—Intestinal anthrax; anthrax septicemia; hemorrhagic leptomeningitis; anthrax edema of the scalp, neck and chest; hemorrhagic edema of the omentum, mesentery and tissues of the mediastinum; subpericardial hemorrhages; edema of the glottis; edema and hyperemia of the epiglottis; hemorrhages into the tissues of the mesentery; submucous gastric hemorrhages; hemorrhagic mesenteric lymphadenitis; acute splenitis; hydroperitoneum, thorax and pericardium; dilatation of the left ventricle of the heart.

Brain.—There was no injury of the outside of the skull. The pia arachnoid tissues of the vertex of the brain were filled with a bloody fluid which almost completely obscured the convolutions and was most abundant over the upper and lateral surfaces of the cerebrum, but present also though less abundant

on the base of the brain and the cerebellum. The arteries at the base of the brain were collapsed completely.

Bacteriology.—In the stained smears from mesenteric lymph glands and spleen, large gram-positive square-end, rod-shaped bacilli in long chains and filaments were seen, in the former in enormous numbers, in the spleen relatively few. Cultures from the spleen and mesenteric lymph glands yielded bacilli similar morphologically to those seen in the smears, identified as Bacillus anthracis, and in the latter there were a few colonies of Bacillus coli as well.

The brain was received in formalin 3 hours after it had been removed, and it was still very soft. After a few days it was cut and no grossly demonstrable hemorrhages were found in its substance. Many of the vessels in the outer layers of the cortex and the striate vessels were distended with blood.

Fig. 5.—Early lesions in the adventitia of a leptomeningeal artery (case 3), lumen to the right, showing a vas vasorum (a) containing three red blood cells and two bacilli, a place of focal edema (b) with two bacilli in it, red blood cells (c) outside the lumen of the vas vasorum and (d) loose tissue of the adventitia; Gram-Weigert stain. X 700 diam.

Microscopic Examination.—In the spaces of the pia arachnoid there were large numbers of red blood cells and a few bacilli. In the meshwork of the pia arachnoid, besides these same elements, there were a few leukocytes, mostly mononuclears. The pia mater was a little thickened by a moderate amount of leukocytic exudate and bacilli (fig. 4) though to a less degree than in either of the other brains. The hemorrhagic leukocytic bacillus-laden exudate was continuous into the brain substance by way of the adventitia of the cerebral vessels in exactly the same way though to a less degree than in the other two brains. As in brains 1 and 2 the pia mater was in places dissected away from the brain by red blood cells which also partly filled the perivascular spaces of the vessels which pierced the pia. No thrombi were found in any of the cerebral or leptomeningeal vessels. There were numerous bacilli in the adventitia of the larger cerebral veins associated in places with patches of edema

and dissection in some of which places the walls were entirely broken through. In the leptomengingeal vessels of small (less than 1/16 mm. in diameter) caliber there was no change except for an occasional bacillus in the adventitia.

One leptomeningeal artery, 0.5 mm. in diameter, was cut obliquely, almost longitudinally, and the vasa vasorum could be seen distinctly and traced into its media. There were numerous bacilli scattered along the walls of these tiny vessels and an occasional bacillus in the lumen (fig. 5a). More bacilli were counted in the lumen of one of these channels in one section than were found in the interior of all the larger vessels of the entire series. In the fork of the branch of one vas vasorum, outside of the channel, there was an irregularly oval, pink granular place about 20 mikrons in diameter containing 2 bacilli (see fig. 5b). There were 5 or 6 red blood cells outside this place in the delicate tissue of the adventitia (fig. 5c). These blood cells were also outside of the vessel channel. On account of the delicacy of the wall of this vas vasorum no definite discontinuity could be demonstrated. There were

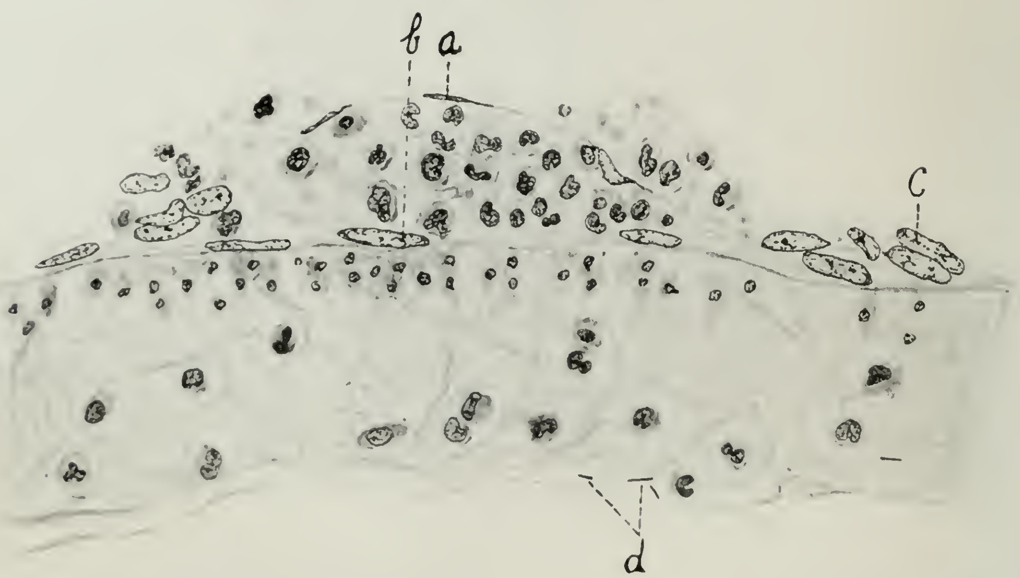

Fig. 6.—Subintimal infiltration of leucocytes; a, b and c, nuclei of endothelial cells; d, bacilli in a minute vas vasorum of the adventitia.

many other similar granular places of edema where the fibers of the adventitia of this and other vessels were widely separated. Some of the endothelial cells of the lining of the larger vasa vasorum were large and a few of them had swollen bacilli in their protoplasm. When a part of a chain of bacilli lay within and a part outside such a cell those within were swollen and those outside had their normal size and shape.

Careful search through the literature has yielded reports of 45 cases of anthrax with grossly demonstrable lesions in the brain: Bruce and Shennan [3] in their report of one case with leptomeningeal hemorrhage were able to collect 26 others. In addition to these Weichsel-

[3] Rev. Neurol. and Psychiat., 1910, 7, p. 521.

baum[4] reported 2 cases; Burmeister,[5] 2; Meyer,[6] 1; Wepfer,[7] 2; Wagner,[8] 1; Reece,[9] 1, and Greenfield,[10] 3. Reports of other cases are referred to elsewhere in the text. Of these 45, sufficient details are given in 29 to note the following: average duration of illness, 2 or 3 days; malignant pustule of the head or neck, 17; intestinal and pulmonary, 7; malignant pustule of the extremities, 3; edema of the neck, 2; hemorrhage into the meninges, 20; hemorrhage into the meninges and brain substance, 9. In only one of the 7 intestinal and pulmonary cases did hemorrhages occur in the brain substance.

In every reported case in which the details are given there were symptoms of cerebral irritation coming on usually several hours before death, and sometimes even 1 to 5 hours before. The great majority of deaths were ascribed to the cerebral lesions, whereas in those with delayed cerebral symptoms death was invariably said to be due to other lesions, notably edema of the larynx. In any event, however, the minds of those with cerebral lesions do not remain clear up to the time of death and it was even suggested by Bell [11] that whenever symptoms of cerebral irritation appear in the course of an anthrax infection, it is most probably due to brain lesions. There are several cases recorded with the first symptoms meningitic, a rather sudden onset with general malaise, headache and often dizziness. Nausea, vomiting, chilliness, pains in the back and extremities and restlessness soon follow. In less than 24 hours the patient may become delirious. This is usually accompanied by convulsions, which may be so violent as to require restraint; then coma and death. Difficulty in swallowing and breathing may appear early and occurs most commonly when there is a malignant pustule or edema of the neck. Muscular twitchings may be present very early. The fever is high at first, 100 to 105 F., later falling to 99 degrees or even lower. Leukocytosis is the rule and may be as high as 75,000 per cu mm. When spinal puncture was made blood tinted fluid was obtained, which was loaded with bacilli. There is one recent report [11] of a recovery from anthracic meningitis where the diagnosis was made by spinal fluid examination. These spinal fluid findings are so constant that in every case in which blood stained

[4] Internat. Klin., Rundschau., 1888, 2, p. 1369.
[5] Inaug. Dissertat., Rostock, 1907.
[6] Deutsch. med. Wchnschr., 1901, 40, p. 1027.
[7] Inaug. Dissertat., Heidelberg, 1910.
[8] Archiv. der Heilkund, 1874, 15, p. 1.
[9] Lancet, 1917, 1, p. 409.
[10] Local Gov, Bd., Med. Officer Reports, London, 1881.
[11] Czyhlarz, Ernst: Wien. klin. Wchnschr., 1916, 29, p. 768.

fluid is obtained smears should be examined immediately and if large bacilli are found the patient isolated until the organism is carefully identified. This identification cannot be too thoroughly carried out, as is shown in one of the cases of this series in which bacilli were found, grown on plates, and discarded as B. subtilis.

There are two routes by which anthrax bacilli may invade the brain and its membrane, the lymphogenous and hematogenous. Risel,[12] Bruce and Shennan,[3] Fulci,[13] and Herzog[14] have reported cases that seem to show that anthrax bacilli may reach the brain through the lymphatics. There is no such direct evidence that anthrax bacilli do localize in the brain by way of the blood stream, though it would be extremely difficult to explain such localizations in any other way in those cases in which the only other focus in the body is in the intestine or lungs or on one of the extremities. Of the 36 cases in which the location of the primary lesion was stated, 17 belong in this last category. Although there may be some definite relation between the location of the primary lesion and brain complications, such a relation is not borne out by the statistics.

Not only from the study here presented but also from the studies of similar lesions by others I am impressed with the importance of recognizing two gross anatomic pictures produced in the brain by anthrax; one, the soft red viscid brain with blood only in the leptomeninges, the other, the soft red viscid brain with multiple hemorrhages of characteristic shape and distribution in its substance. Other observers have failed to emphasize these changes.

The brains of this report are excellent examples of these two types (brain 3, type 1; brains 1 and 2, type 2). It is fair to state for the present that no other disease is known to produce such changes as in type 2, namely, hemorrhages with such characteristic shape and distribution; the alterations are so marked and particular as to be characteristic of anthrax and in this sense useful in the recognition of anthrax by postmortem examinations of the bodies of persons the circumstances of whose deaths are unknown.

The microscopic picture is even more characteristic and Wagner,[8] Fraenkel,[15] Risel,[12] Symmers and Wilson,[16] Fulci,[13] and Herzog,[14] in reporting cases have fairly adequately described these changes.

[12] Ztschr. f. Hyg. u. Infektionskr., 1903, 42, p. 380.
[13] Histol. y. Histopath. Arb. u. d. Grosshirnrinde, 1913 14, 6, p. 161.
[14] Beitr. z. path Anat. u. z. allg Path, 1915, 60, p. 513.
[15] Ztschr. fur Hyg. u. Infektionskr., 1898, 27, p. 315.
[16] Jour Path. and Bacteriol., 1908-09, 13, p. 251.

The most comprehensive work was done by Fulci on a brain with cortical hemorrhages and Herzog on three brains without cortical hemorrhages, both of whom concluded that the vascular lesions were produced by agents acting outside of the vessel lumen. Neither of them found bacilli in the vessel lumens. Such changes as occur early in the walls of the leptomeningeal vessels were not described by any of these investigators.

The essential microscopic picture is one of acute inflammation plus hemorrhage. The large amount of hemorrhagic exudate and almost complete absence of retrogressive changes is in strict accord with anthrax lesions elsewhere in the body. The large vessels of the leptomeninges seem to be the first to show this change and of these the veins are affected most. The changes in brain 3 are evidently of shorter duration than those in brains 1 and 2 on account of the relatively larger numbers of red blood cells, small numbers of leukocytes and bacilli in the exudate, the small degree of extension of this exudate into the brain substance along the cortical vessels and the relatively small amount of edema in the walls of the cerebral vessels, especially the cortical. In brain 2 the inflammation is probably of longer duration than in either brain 1 or 3, because the leukocytes, fibrin, and bacilli of the exudate predominate; there has been more and deeper extension into the brain substance along the vessels and there is much more thrombosis of the cortical vessels. The changes in brain 1 are also of considerable duration, but the exudate around the cortical vessels is much more hemorrhagic and there is relatively little thrombosis. These differences in the inflammatory process of the brain are in strict accord so far as one can judge with the duration of brain involvement; although in the third case no account of cerebral symptoms was obtained.

The amount of cortical changes seems to depend somewhat, therefore, on the duration of the inflammatory process and the extension of the exudate from the leptomeninges into the brain substance along the vessels; this I believe explains why in some of these cases there are no intracerebral hemorrhages. Such an extension inward of the exudate, especially of the red blood cells, is facilitated in the case of the striate vessels by the large channels in which these vessels lie.

It must be remembered that the microscopic study of these brains was necessarily limited to relatively small portions and that if similar studies could be made of all parts we might possibly come nearer to

the truth. But so far as one can judge with the unaided eye and by taking sections not in series from other regions, the changes described are representative of the whole.

SUMMARY AND CONCLUSIONS

A number of cases of anthrax have occurred in Chicago recently, many of them among workers in the "curled hair" industry. In at least three of these cases there were brain complications and in two of the three anthrax was not suspected until after death. It seems reasonable therefore, to suspect that from time to time similar cases may go unrecognized. In an effort to eliminate such a possibility a careful microscopic and bacteriologic study should be made of every bloody spinal fluid, provided one is certain that the blood is not a contamination; and especially should this be done if there are symptoms of cerebral irritation.

These cases can be easily recognized at necropsy by the soft, red viscid brain, especially if there is a history of an acute illness not resulting from trauma; and it must be remembered that there may be one of at least two types of gross changes—either the brain with the characteristic intracerebral hemorrhages or the brain with blood only in the membranes. On account of the large quantity of blood which is sometimes present it would be easy for one who had not seen such brains to attribute the cause to trauma, but this may be avoided by recognizing the inflammatory quality of the changes and by checking up with stained smears of the exudate.

The lesions in the brain in anthrax are essentially extravascular, meningo-encephalitic, and the acute hemorrhagic nonpurulent nature of the inflammation with destruction of tissue by edema is similar to that produced by anthrax in other places of the body. With suitable stains large numbers of bacilli may be found in these lesions.

The infection of one of the brains of this series (Case 3) was evidently by the hematogenous route, first, because the primary focus was so far removed (intestine) and second, because of the finding of small bacillus-containing foci of edema and dissection along the vasa vasorum of the leptomeningeal vessels.

THE FATE OF INDIA INK INJECTED INTO THE BLOOD

I. GENERAL OBSERVATIONS

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Knowledge of the fate of foreign bodies introduced into the blood will throw light on the distribution and fate of microbes that invade the blood. In the latter part of the last century many investigators [1] made studies in this field by means of cinnabar, ultramarine blue, etc., and the results agreed in so far as the particles were arrested in the spleen, marrow, liver and lymph nodes, and mostly seen in extravascular connective tissue cells. My study concerns India ink, which consists of smaller and lighter particles than the other substances that have been used before. Some of the results were given before the Japanese Society of Internal Medicine in 1913 [2] and 1914.[3] The results obtained by Kusama [4] and also by Kiyono [5] agree in that India ink injected into the blood is deposited principally in the endothelial cells of the liver, marrow and spleen, and in the latter organ in splenocytes, while later the granules accumulate irregularly in the organs mentioned.

METHODS

A good stick of india ink is rubbed on an ink stone with a small amount of 0.8% salt solution, and this process is repeated until a considerable quantity of suspension is obtained, which is centrifugated for about 20 minutes and the upper part of the suspension filtered several times through the same paper. After sterilization by steam discontinuously 3 or 4 times, the suspension is kept closely sealed in the icebox. In my work rabbits and guinea-pigs were used and the amount injected as a rule was 0.4 c c per kilo of animal, but of course

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[1] Ponfick. Virchows Archiv, 1869, 48, p. 1. Hoffmann and Langhans, ibid., p. 303. Siebel, ibid., 1886, 104, p. 514.

[2] Nippon Naikagakkai Zassi, 1913, 1, p. 185.

[3] Ibid., 1914, 3, p. 5.

[4] Ziegler's Beiträge, 1913, 551, p. 459.

[5] Die Karminspeicherung, Jena, 1914. Kyoto Igaku Zassi, 1917, 14, p. 198.

the actual quantity of ink injected cannot always be standardized accurately, but I took especial care to prepare enough suspension at one time for the same series of experiments. The animals were killed (blow on neck or air embolus), and all the organs and tissues were removed immediately and fixed in Zenker's fluid or formalin. The marrow was taken out carefully from the femur while certain short and flat bones were decalcified in 2% nitric acid for the examination of compact bone, joint tissue and other structures. The spinal cord and sympathetic nerves were also examined at several places. After embedding in paraffin, sections were made 5 mikrons thick; in certain cases thicker sections were used. Giemsa and Wright stains were used for the blood; hematoxylin and eosin gave the best results in the study of the ink cells and in this case the tissues were fixed in alcohol ether and destained with 0.002% HCl in 60% alcohol in order to avoid laking the blood corpuscles. The eosin staining was purposely made rather light. The only disadvantage of this method is that the granules in mast cells are not stained. It was found that Ehrlich's triple acid stain gave the best results in general.

EXTERNAL APPEARANCES

When a certain amount of ink has been injected, the conjunctiva and other visible mucous membranes as well as the skin are colored black, but after some time the color returns to normal.

NUTRITION

Reasonable amounts of ink suspension injected as described do not seem to be directly injurious to the animals. They increased in weight and size, brought forth young, their behavior in every way not differing from that of perfectly normal animals. After the injection of a large amount, e. g., over 20 c c per kilo of rabbit, no symptoms would develop, but after some days the animals would become thin and sometimes die, though that was not always the case. Rapid injection, especially of highly concentrated ink, might cause paralysis and frequently death, and in the organs of such animals the ink did not mix with the blood but formed definite emboli. The maximal dosage for rabbits was 35 to 45 c c. Guinea-pigs often died after receiving 6 to 7 c c of ink suspensions (12 to 14 c c per kilo); they evidently are less resistant to such injections than rabbits.

THE BLOOD

For a while after the injection ink granules were present in the blood, usually for about 3 hours after the usual dosage, and for about 6 to 12 hours after the injection of a larger amount. In a short time ink granules appeared in the polymorphonuclear and large mononuclear leukocytes, such granules being found only occasionally in the large lymphocytes and never in the small. In the eosinophile cells I have not found any granules in the experiment now described; the same is true of mast cells, which increase some time after the injection, but

TABLE 1

PERCENTAGE OF INK CELLS AFTER INJECTIONS OF INK

Rabbits	Cells	3 Hrs.	6 Hrs.	12 Hrs.	24 Hrs.	2 Days	4 Days	7 Days	14 Days	21 Days	1 Mo.	2 Mos.
I 1905 gm. 30 c c ink suspension	Polymorphonuclear ink cells	17.3	21.8	24.2	19.8	4.9	3.9	0	0	0	0	
	Mononuclear ink cells	10.0	23.0	38.0	5.0	18.8	24.2	3.5	0.7	0.4	3.1	2.0
	Large lymphocytes	0	0	0	0	1.0	1.0	0	0.6	0	0	0
	Nucleated red cells	18.0	101.0	89.0	28.0	10.0	7.0	6.0	2.0	1.0	0	
II 1775 gm. 25 c c ink supsension	Polymorphonuclear ink cells	....	29.0	35.1	19.3	3.0	1.1	0	0	0	0	0
	Mononuclear ink cells	....	7.8	36.2	64.4	25.1	13.0	4.9	2.5	0	3.2	3.6
	Large lymphocytes	....	0	0	0	0	0	0	0	0	0	0
	Nucleated red cells	....	109.0	90.0	31.0	10.0	7.0	8.0	3.0	0	1.0	
III 2055 gm. 7 c c ink suspension	Polymorphonuclear ink cells	7.8	18.5	17.0	12.0	2.4	0.5	0	0	0	0	0
	Mononuclear ink cells	21.2	7.1	0	4.9	6.4	0.7	2.9	1.9	0	2.8	1.8
	Large lymphocytes	0	0	0	0	0	0.4	0	0	0	0	0
	Nucleated red cells	21.0	61.0	45.0	12.0	4.0	3.0	1.0	1.0	3.0	0	
IV 2605 gm. 9 c c ink suspension	Polymorphonuclear ink cells	4.2	14.3	11.1	2.4	0.5	0	0	0	0	0	0
	Mononuclear ink cells	19.0	0	17.0	2.4	5.0	6.5	3.2	1.7	0	0.6	1.0
	Large lymphocytes	0	0	0.8	0	0	0	0	0	0	0	0
	Nucleated red cells	....	76.0	52.0	14.0	4.0	4.0	1.0	0	0	0	

The number of nucleated red cells indicates the number of such cells for each 500 leukocytes.

even on reinjection of ink at this time no granules were found in these cells. Nucleated red cells appeared in the blood in considerable numbers, especially after injection of large amounts of ink suspension. The table shows the percentage of ink containing cells and the number of nucleated red cells for each 500 leukocytes in various stages after the injection. When large amounts were injected, the polymorphonuclear ink cells were most numerous 12 hours after the injection, and after the usual amount their maximum was reached in about 6 hours. In both cases the number decreased markedly between 24 and 48

hours after the injection, and in 4 to 7 days all such ink containing cells had disappeared as a rule; in one instance they persisted for 3 weeks. The mononuclear ink cells fluctuated much more, but were present in fairly good numbers for 4 to 7 days, when a marked drop occurred, some however persisting for even over a year, and possibly during life. One rabbit showed one mononuclear ink cell of 74 counted and another one in 192, one year after the injection. In late periods the granules in mononuclear cells were always few. These results accord quite well with those obtained with cinnabar by Ponfick and by Hoffmann and Langhans. The number of granules in the polymorphonuclears usually varied from 1 to 30 or more, the cells sometimes being filled to distention. In all cases the granules were scattered through the cytoplasm, appearing only occasionally in the nucleus.

Shortly after the injection a considerable number of nucleated red cells and polychromatophile red cells appeared in the blood. Kusama made similar observations. The number of such cells was at a maximum in 6 hours, and then there was a gradual decrease, but the cells could be found 4 and 7 days after injection. With larger doses of ink, various abnormal cells made their appearance, often containing ink granules as did also the nucleated red cells. Schulze,[6] Arnold[7] and others found granules in the red corpuscles of the frog treated with methylene blue. Rost[8] found that corpuscles in frogs subjected to vital staining would contain granules more frequently when some injurious agent was injected. Recently Kiyono and Chuin[9] stated that primitive erythroblasts of higher vertebrates are capable of a slight degree of phagocytosis. Hence, it seems not unlikely that the phagocytosis of ink granules may be the result of acute regenerative processes in connection with the red cells. The relation of these red cells to so-called pseudolymphocytes and other questions are reserved for further investigation.

INTERNAL ORGANS

After death the liver, spleen and marrow were intensely black. Some lymph nodes, especially about the stomach and along the jugular vein, were also blackened, and this is true as well of the omentum and periosteum, especially after large doses and late after the usual doses of ink. Sometimes the small intestine and the lungs were stained

[6] Anat. Anzeiger, 1887, 2, p. 684
[7] Virchows Arch., 1899, 157, p. 429; Arch. f. Mikr. Anatomie, 1898, 52, p. 523.
[8] Arch. f. d. gesam. Physiol., 1911, 137, p. 359.
[9] Nisshin Igaku, 1918-19, 8, p. 475.

blackish after large doses. In the case of the periosteum, it was usually in the long bones (femur, tibia, ribs) that it showed blackening, usually some weeks or months after the injection. In the flat or short bones such a change was less marked, the scapula, however, often showing some staining.

In the liver ink granules were deposited in the endothelial cells of the blood capillaries, in the earlier stages in regular distribution giving a radial arrangement under low power from the central vein to the periphery of the acinus. Under higher power the content of different cells in granules varied, some cells being stuffed with granules and roundish from having drawn in their processes. Such cells often look as if about to fall off into the blood. Kiyono noted this also. The ink granules tended to accumulate around, or at the end of, the nucleus and in the early stages the granules were round and smooth but later they became irregular and apparently formed aggregates. Glisson's capsule and the liver cells proper did not contain any granules in the early stages after the usual dosage. About a week after the injection the amount of granules seemed to increase in some of the cells and collections of such endothelial cells began to form, the distribution of the granules being much more irregular than at first. In late stages there were large masses of cells filled with granules, such masses apparently increasing in size at the same time as granules containing cells from other parts of the liver decreased. After a year or so such masses might be detected with the naked eye when the slides were held against the light and in such cases there were only a small number of phagocytic cells in the substance of the liver elsewhere. In the course of their accumulation, the ink granules appeared to proceed from the center to the boundary of the acini, hence in the later stages the granules were found mostly at the border or in Glisson's capsule. Usually it took several days or a week before the granules appeared in Glisson's capsule. Giant cells were often found in and about accumulations of granules, but no special cell proliferation seemed to develop around the masses except that late in the process Glisson's capsule seemed to become somewhat thicker. After large doses of ink, granules would accumulate more quickly and even enter liver cells, and this seemed to be the case especially when several injections were made into the same animal. In two healthy rabbits ink granules appeared within the liver cells, in one case, the rabbit weighing 2,150 gm., after injection of 4 c c of ink suspension every day for 6 days and in the case of the second animal, which weighed 2,000

gm., after the injection of 7 c c of suspension on 4 different occasions in the same week. Kiyono reports a similar observation. Feeble rabbits, afflicted with coccidiosis, frequently presented an irregular distribution of endothelial cells and also the formation of giant cells by the coalescing of endothelial cells, resulting not only in an irregular distribution of the granules from the first, but also in the appearance of granules in the liver cells at an early period.

In the liver cells the granules were usually smaller and more uniform in size than in the endothelial cells, usually being scattered in the body of the cells. Such phagocytic liver cells were distributed irregularly.

In the spleen the endothelial cells of the capillaries and sinuses, as well as the splenocytes, took up ink granules after the usual dose. The malpighian bodies were practically free from ink in the early stages, but in feeble animals or after large doses of ink there would be some granules here also. The granules in the spleen seemed to vary in size more than those in the liver, and in the spleen also the granules subsequently migrated and eventually accumulated in larger masses so that after a year or so the masses could be seen with the naked eye. There seemed to be no special place in which such masses would form by preference. Giant cells of the foreign body type and large phagocytic cells were present in such masses, but there was no special cellular changes at their periphery. The spleen seemed to acquire more granules as time went on while in the marrow as well as in the liver the amount of ink granules seemed to decrease. In sections of the spleen of animals killed immediately after the injection of ink the capillaries could be traced easily by means of the ink granules in the endothelial cells. In the malpighian bodies a few capillaries were found in this way, but the endothelial cells here seemed to have less avidity for the ink. In the central artery there seemed to be almost no phagocytosis. The plasma cells present in small numbers in the spleen gave no evidence of phagocytosis. In special cases of animals injected with streptococci or streptococcus products, there was a marked precipitation of granules in the borders of the malpighian bodies, suggesting that phagocytic cells may develop from the reticulo-endothelial cells and passing from the center toward the periphery.

In the marrow the ink granules were deposited first in the endothelial cells of the capillaries and veins. In the red marrow, which is rich in veins, the venous network was outlined distinctly. In the early stages no granules were found in the marrow cells proper, but

later the granules formed accumulations in masses, just as they did in the liver and spleen, the endothelial cells gradually becoming free. At this time the reticular cells contained granules. The osteoblasts and osteoclasts also took up granules, especially after large doses, but the myelocytes and megakaryocytes remained free.

As a rule, the bone tissue was free from granules in the earlier stages after the usual dosage but later ink granules appeared not only in the Haversian and the Volkmann canals, but also in the bone cells, although to a limited extent only, the granules being small in size and round.

At first the periosteum remained free from granules, but after a time granules were deposited to such an extent as to be detectable with the naked eye, and microscopically the granules were contained in the endothelial cells especially of the lymph vessels. After large doses the deposit occurred earlier and in greater degree.

Ink granules were also deposited in the vertebrae and in the scapulae.

Granules were found in the endothelial cells of the suprarenals, the amount depending on the amount of ink suspension injected. In from a few weeks to months the amount of granules in the suprarenals seemed to increase, the fascicular zone containing perhaps the most. After this they accumulated more particularly in the medulla, in which small collections formed. Feeble rabbits and rabbits injected with streptococci seemed to contain early more granules in the suprarenal than normal animals.

In the kidney granules were deposited to a limited extent in the cortex, especially in the glomeruli, but only after considerable doses. In late stages the granules had disappeared, except for traces in the glomeruli.

In the lungs, after moderate doses of ink suspension, granules were found in the endothelial and mononuclear cells, but no ink emboli were found except after large injections. In these organs the amount of granules found was always rather small and only a few masses. Granules were found also in the epithelial cells of the alveoli, but only occasionally. Eventually the granules of ink disappeared almost wholly from the lungs.

In the early stages and after the usual dose, the omentum and peritoneum commonly remained free from ink. In omentum majus ink granules often appeared in the middle and later stages and in

feeble rabbits as well as after large quantities deposits also occurred earlier. The granules here were located in the surface cells and also in the clasmatocytes. In two rabbits with coccidiosis and ascites ink granules appeared in the abdominal cavity, the ascitic fluid becoming blackish in a short time after the injection of 25 and 30 c c of suspension; in healthy animals the granules appeared only in the endothelial cells of the capillaries even after large doses. The results in feeble animals, after intravenous injection, resembled very much the result after the intra-abdominal injection of a small amount of ink. In later stages the granules gathered in the perithelial tissue and surface cells, forming accumulations without any definite arrangement except about taches laiteuses.

Ink granules also appeared, but only to a limited extent, in the endothelial cells of the blood vessels in general, mostly in the venous capillaries. The cells of the endocardium frequently contained granules, especially in the middle periods after the injection, being situated usually about the ends of the nuclei, very small and as a rule round. In the capilaries of the myocardium granules were found, as a rule, but in the later stages they disappeared.

Lymph Nodes: Sometimes a small quantity of ink appeared in the early stage after the usual dose, and later there was often found a greater accumulation, especially in certain nodes such as the cardiac, hepatoduodenal and along the jugular. These nodes were often quite black even months after the injection. The granules were deposited in groups in the cortex as well as medulla and in such masses were found giant cells, mostly at the outskirts. At first the granules appeared in the endothelial cells of the capillaries and lymph sinuses but only when large doses were injected.

Of all the capillaries, those of the villi of the intestine seem to have more ink granules in their endothelial cells and in consequence after a large dose of ink suspension the small intestine was as a rule blackish.

In the ovary and testis a small number of ink granules were found after the usual dose. In the ovary the granules appeared in the stroma and theca folliculi; increasing somewhat with time, but I failed to find any granules in the follicular cavity. In the testis the endothelial cell as well as connective tissue cells were found to contain a moderate number of granules some time after injection, but even after large doses it was only late that a few granules were detected

in the interstitial cells. Foa [10] ascribes phagocytic action to this cell while Ishibashi [11] differs on the basis of the results of injecting an emulsion of soot directly into the tissue. I shall make a special report concerning this.

In two cases I found granules in the placenta, the animals having been injected with large doses of ink suspension (1 rabbit, 1 guinea-pig). In the fetus and new-born of mothers injected with ink suspension (4 rabbits and 2 guinea-pigs) I did not find any granules in the liver or spleen.

Soon after injection clasmatocytes and connective tissue cells did not contain any granules, but later granules were often found in these cells in various parts of the body. After the injection of large doses granules appeared earlier in the cells.

In the cells proper of the brain, spinal cord, hypophysis, muscle pancreas, thyroid, salivary gland, sympathetic ganglion, etc., I failed to find any granules at any time.

When the ink is injected into the blood, granules are attracted first to the endothelial cells of the liver, the spleen, and the marrow and to the splenocytes, which cells quickly take them up. If the quantity injected is small, these cells rapidly dispose of all the ink granules. If the quantity is larger, the cells are not able to take up all the granules at once so that some remain in the blood. In the case of the largest quantity injected in these experiments, granules remained in the blood for about 12 hours. Apparently we have here an example of the phagocytic limit being reached by these cells. Other cells, such as those in the suprarenals, kidney, intestines, etc., become more phagocytic under these circumstances. When the injection of ink is repeated a wider range of cells appear to become engaged in phagocytosis. Some time after the injection accumulations of ink granules form, relieving some of the phagocytes at the same time as new phagocytic cells are formed.

In feeble rabbits replacement and accumulation of ink granules takes place more quickly than in healthy rabbits, due perhaps to degenerative and regenerative changes in phagocytic cells. It is also noticeable that the granules reached the lymph spaces more readily in feeble rabbits, as illustrated especially by the changes observed in the omentum and in ascitic fluid. The entrance of granules into liver cells may occur under the same conditions.

[10] Quoted by Ishibashi.

[11] Mitth. ad. med. Fakultät d. kais. Univ. 3, Tokio, 1919, 22, p. 39.

Earlier investigators (Ponfick with cinnabar, Hoffmann and Langhans with cinnabar and ultramarine blue, and Siebel with indigo) observed that after intravenous injection foreign particles were deposited outside the vessels in the liver, spleen, marrow and lymph nodes. Ponfick urged that the deposition takes place directly in special cells outside the vessels, while the others asserted that there is intermediary action on the part of phagocytic leukocytes from the blood and even suggested that these leukocytes change into connective tissue cells. Kiyono ascribed an eliminating function to the liver and the other organs mentioned, foreign particles reaching the lymph spaces through the capillary walls. I agree with this view with some modification, which I shall discuss later.

The granules of ink injected into the blood seem to have no definite relation with any excretory or secretory organs and careful repeated examination of the urine and bile has not revealed any granules. After the injection of large doses, especially in feeble rabbits, granules often appeared in the liver cells proper but without any relation to the bile capillaries. It would seem as advocated by Kiyono, that ink granules can only be removed from the body by way of the leukocytes.

SUMMARY

When a suspension of India ink is injected into the blood the mucous membranes and spleen become black and remain so for variable lengths of time, depending on the amount injected.

Free granules are present in the blood for several hours when large quantities are injected, but disappear sooner after smaller quantities.

Ink granules are deposited regularly in the endothelial cells of the liver, spleen and marrow and in the splenocytes, and are eventually gathered together in accumulations of considerable extent. In the liver the granules are passed along from the centers to the borders of the acini and into Glisson's capsule. In the spleen the granules are accumulated irregularly, often at the outskirts of, or in, the malpighian bodies. In the marrow there is no special arrangement of the granules. In the peritoneum and in the osteal canals, granules appear in the later stages after the injection, some perhaps reaching the lymph spaces from the marrow. The granules found in the bone cells were very small. After large injections osteoblasts and osteoclasts may take up granules. Only a small number of granules appear in the suprarenals, kidneys and lungs. In the earlier stages there usually are few gran-

ules in the lymph node but after some time granules appear regularly in certain nodes (cardiac, hepatoduodenal, jugular) which may be quite black. Few granules are generally deposited in the blood vessels and more in the venous than in the arterial vessels. After large doses granules are found in the endocardial cells.

In the liver and spleen, as well as elsewhere, large phagocytic cells may appear, stuffed with granules, but there is no special connective tissue proliferation about accumulations of granules, in which giant cells may be found.

After large doses or repeated injections granules may appear in the liver cells proper and in the medulla of the lymph nodes, and in time aggregations of granules may form here and in the malpighian bodies of the spleen. In rabbits with coccidiosis granules may appear in the liver cells earlier than in normal animals. Only comparatively few granules are deposited in the omentum except when large quantities of ink are injected, or when the rabbit is feeble. In rabbits with ascites granules may pass into the fluid. In the ovary granules may appear in the stroma and in the theca folliculi and in the testis the interstitial cells may contain occasional granules. After large doses granules may appear in the placenta, but I did not find any ink granules in the fetus.

In late stages after the injection connective tissue and other cells in various parts of the body may contain granules, but without any regular distribution. Granules were not found in the parenchyma cells of the nervous system and various other organs with the exception of the liver and spleen.

In the blood polymorphonuclears and mononuclears take up granules; large lymphocytes do so only occasionally. The polymorphonuclear ink cells increase rapidly after the injection, reaching the maximum in 6 hours after small doses and in about 12 hours after large doses, disappearing gradually in the course of several days. The mononuclears pursue a more irregular course, such cells apparently being set free from the liver and spleen, but by the seventh day the number is greatly diminished; however, small numbers of such cells with ink granules in them may occur in the blood for as long as a year or more and perhaps during the rest of the life of the animal. After the injection a considerable number of erythroblasts appear in the blood, especially after large doses; the number continues to increase for about 6 hours when there is a rapid drop, normal conditions being reached in about a week or so. Various abnormal cells also appear

in the blood, often containing ink granules, and sometimes granules are found in erythroblasts. The eosinophiles and mast cells do not take up granules.

With careful preparation of the ink suspension and care in injection, the health of the animal injected need not be disturbed, but rapid injection of large doses or highly concentrated suspension may cause death promptly, or if the animal survives it may remain feeble for several days. In such cases the animals may not live as long as those injected with smaller quantities.

Apparently ink granules are not discharged from the body by any particular organ after injection into the blood, but it is possible that they are carried to the outside by emigrating phagocytes.

THE FERMENTATION OF XYLOSE BY BACTERIA OF THE AEROGENES, PARATYPHOID B. AND TYPHOID GROUPS *

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In previous papers we have recorded results which show that xylose is fermented by Lactobacillus pentoaceticus with the production of almost equal amounts of acetic acid and lactic acid, and traces of alcohol and carbon dioxide. Approximately 80-90% of the sugar consumed may be accounted for by the two acids, acetic and lactic. Toward the aldohexose sugars, we noted these organisms behaved in a different manner; the end products are chiefly alcohol, lactic acid, carbon dioxide, and a small amount of acetic acid. Studies have since been made on the products of the fermentation of xylose by bacteria of the aerogenes-typhoid group, the results of which are presented in this report.

Of the various sugars used in fermentation tests to separate typhoid, paratyphoid A, and paratyphoid B, xylose has found especial favor. Weiss,[1] Stern,[2] Teague and Morishima,[3] and others have shown that xylose is fermented by B. typhosus without gas, by B. paratyphosus B with gas, and is not attacked by B. paratyphosus A. These groups of the typhoid organisms may be further subdivided into strains according to variations in the fermentation of xylose. In view of these facts and of the interest attached to the mode of decomposition of xylose, it was considered important to study the principal substances formed in the fermentation of this sugar. A comparison of these results with those obtained with Lactobacillus pentoaceticus presents marked differences in the end products.

The presence of volatile and nonvolatile acids, as well as alcohol, among the products of fermentation of glucose and other sugars by bacteria of the aerogenes-typhoid group has been noted by certain investigators. Harden[4] showed that the chief products of the fermentation of glucose by bacteria of the colon-typhoid group are lactic, succinic, acetic, and formic acids, ethyl alcohol, carbon dioxide, and hydrogen; the proportions of these substances varied with the different organisms. The typhoid bacteria produced a large

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[1] Jour. Med. Research, 1917, 31, p. 135.

[2] Centralbl. f. Bakteriol., I. O., 118, 82, p. 49.

[3] Jour. Infect. Dis., 1920, 27, p. 52.

[4] Jour. Chem. Soc., 1901, 79, p. 610.

amount of formic acid instead of a mixture of carbon dioxide and hydrogen. The colon bacteria produced only small amounts of formic acid but considerable quantities of carbon dioxide and hydrogen. The absence of carbon dioxide and hydrogen in the typhoid cultures is assumed to be due to their inability to ferment formates from which carbon dioxide and hydrogen are produced by the colon group. The lactic acid produced by B. typhosus was found to consist of about 47.5% of active acid. Unlike the fermentation with colon, he found that the growth of the typhoid organism was not vigorous and terminated when about half the sugar was fermented. The limited fermentation of glucose by B. typhosus and the production of both levolactic acid and inactive lactic acid was previously reported by Peré[5] in a paper in which he discussed the fermentation characteristics and products of B. coli and B. typhosus.

Harden and Walpole[6] found that B. lactis-aerogenes produces less acetic acid and more carbon dioxide than does B. coli communis in the fermentation of glucose. From the results of a detailed examination of the products, it was observed that only about two thirds of the carbon of the glucose was accounted for by the products lactic acid, acetic acid, succinic acid, formic acid, ethyl alcohol, carbon dioxide and hydrogen. In a search for other compounds, they found a considerable quantity, approximately 33% of the carbon of the sugar appeared as 2:3 butyleneglycol and a very small amount of acetylmethylcarbinol. In a later report by Harden and Norris,[7] it was found that B. lactis aerogenes produces acetylmethylcarbinol and 2:3 butyleneglycol in the fermentation of glucose, fructose, mannose, galactose, arabinose, isodulcite, mannitol, or adonitol.

Duchacek[8] studied the products of the fermentation of glucose by B. coli and B. typhosus in cultures exposed to the atmosphere and to an atmosphere of hydrogen. In agreement with Harden and his associates, Duchacek found that the colon organism ferments glucose much faster and much more completely than the typhoid. In cultures 11 days old exposed to the air, about 53% of the glucose was fermented by B. coli and about 18% by B. typhosus. In the cultures exposed to the air the fermentation proceeded faster than in an atmosphere of hydrogen. The chief products of fermentation by these organisms were two organic acids, lactic and acetic. With B. coli, carbon dioxide was produced in large amounts in addition to the acids. According to Sera[9] the fermentation of glucose by B. typhosus results in the production of acetic acid, formic acid, and a trace of alcohol. No attempt was made to measure other products of fermentation.

EXPERIMENTAL

The fermentations were in every case carried out in a 300 c c Erlenmeyer flask connected to carbon dioxid traps; 250 c c of culture medium was used. The exact arrangement of the apparatus and the methods of examining the products of fermentation have previously been described in detail.[10] The medium consisted of a fresh yeast water extract, containing 0.5% dibasic potassium phos-

[5] Ann. Inst. Pasteur, 1892, 6, p. 512.
[6] Proc. Roy. Soc., Series B, 1906, 77, p. 399.
[7] Proc. Roy. Soc., Series B, 1912, 84, p. 492.
[8] Centralbl. f. Bakteriol., I, O., 1904, 37, p. 161 and p. 526.
[9] Ztschr. f. Hyg. u. Infektionskr., 1910, 66, p. 162.
[10] Fred, E. B.; Peterson, W. H., and Davenport, Audrey, Jour. Biol. Chem., 1919, 39, p. 347; 1920, 41, p. 431.

phate and 0.5% of Difco peptone. To this was added 2% of xylose and the medium was then sterilized in the autoclave at 15 lbs. pressure for 30 minutes. At the time of inoculation, a few drops of bromocresol purple were added and the cultures incubated at 37 C. The acids formed during the fermentation, as shown by the indicator bromocresol purple, were neutralized with sterilized 1 N NaOH. In all experiments uninoculated controls were made and the results of their analyses were substracted from the results of the inoculated flasks. Although the inoculated cultures without sugar showed a certain amount of fermentation, it was decided not to subtract these results from those of the sugar cultures. Harden [4] and others have shown that the fermentation of peptone in the absence of sugar is different from that in the presence of sugar.

The chief products formed by the action of B. aerogenes, culture 26, B. typhosus, and B. paratyphosus B on xylose are given in this report. The strains of B. typhosus and B. paratyphoid B used were furnished by the Army Medical School. The B. lactis aerogenes and culture 26 were taken from our laboratory stock cultures. These two organisms were isolated from silage and are no doubt closely related strains. The chief biologic characteristics of B. lactis aerogenes and of culture 26 are clearly seen in the following table in which + means acid and gas; and — no fermentation.

TABLE 1

Biologic Characteristics of B. Lactis Aerogenes and Culture 26

	B. lactis aerogenes	Culture 26		B. lactis aerogenes	Culture 26
1. Arabinose	+	+	11. Raffinose	+	+
2. Xylose	+	+	12. Melezitose	—	—
3. Rhamnose	+	+	13. Mannitol	+	+
4. Glucose	+	+	14. Glycerol	—	—
5. Fructose	+	+	15. Salicin	+	+
6. Galactose	+	+	16. Esculin	+	+
7. Mannose	+	+	17. Inulin	—	—
8. Sucrose	+	+	18. Starch	—	—
9. Lactose	+	+	19. Sodium lactate	—	—
10. Maltose	+	+			

These two organisms show a close resemblance to each other; they are rod forms, motile, gram-negative, nonliquefying, and give a positive Voges-Proskauer reaction. They ferment carbohydrates vigorously with the production of much alcohol, carbon dioxide, and hydrogen. The reaction of the medium becomes acid and later reverts to a lower degree of acidity.

The cultural reactions of the four organisms—culture 26, B. lactis aerogenes, B. typhosus, and B. paratyphosus B—were studied in fermentation tubes containing the xylose-peptone-yeast water. Since gas measurements in these tubes, especially carbon dioxide, are not satisfactory, the usual method of procedure was modified. Twenty-four hours after the tubes were inoculated, about 2 c c of sterilized mercury was added; just enough mercury to seal the tube at the lowest point, is sufficient. This modification of the Smith fermentation tube was tested with many different organisms. It was found that the addition of mercury checked the diffusion and escape of carbon dioxide from the fermentation tube. The presence of mercury in these tubes exerts a slightly retarding effect on the growth of the bacteria and it is, therefore, advisable to allow the inoculated cultures to grow for at least 24 hours before adding the mercury. The effect of mercury on the retention of carbon dioxide may be seen from the following results:

	CO_2 from 12 c c of culture
No mercury	0.0222 gm.
Mercury, 2 c c	0.1000 gm.

In this test, the tubes of 2% glucose yeast water were inoculated with a pure culture of pentose fermenters and 24 hours later mercury was added. After 2 weeks the carbon dioxide in the long arm of the tube was fixed with potassium hydroxide and determined by the Van Slyke apparatus. The value of the mercury seal in the retention of carbon dioxide is clearly seen from the results of these analyses. In a similar experiment, the gas retained in the tubes at varying intervals of time was measured.

TABLE 2

Gas Retained in Tubes at Varying Intervals of Time

	Gas Retained After			
	2 Days, C c	6 Days, C c	12 Days, C c	18 Days, C c
Culture 26, no mercury	6.0	7.0	6.0	5.0
Culture 26, mercury	7.0	9.0	9.0	9.0
B. lactis aerogenes, no mercury	4.5	4.5	3.5	2.0
B. lactis aerogenes, mercury	3.5	4.5	10.5	11.0

Here it will be seen that the gas is gradually lost by diffusion through the liquid. In the presence of mercury there is no indication of loss, but often a continued gain in the quantity of gas.

Absorption tests of the gas collected in the closed arm of the fermentation tube showed that from xylose, culture 26 produces about 2 parts of carbon dioxide to one part of hydrogen, while B. typhosus does not produce any gas. On the other hand, B. paratyphoid B decomposes xylose in a manner similar to that of the aerogenes organisms. The ratio of gas is about 2 parts of carbon dioxide to 1 part of hydrogen.

THE FERMENTATION OF XYLOSE BY B. LACTIS AEROGENES, CULTURE 26, B. TYPHOSUS, AND B. PARATYPHOSUS B.

The substances produced by the action of B. lactis aerogenes and culture 26 on xylose were found to be small amounts of volatile and nonvolatile acids, and large amounts of ethyl alcohol, carbon dioxide and hydrogen. The sum of the products, with the exception of hydrogen which was not determined quantitatively, reveals the fact that only about two thirds to three fourths of the xylose is accounted for by these end products. The results of the analyses are shown in the figures of table 3. The yield of volatile and nonvolatile acids was so small that the kind of acid could not be determined. By far the larger part of the decomposed sugar is accounted for by the end product, carbon dioxide. The percentages by weight of the products from the fermentation of xylose by these two organisms are given in table 4.

TABLE 3

THE PRODUCTS OF THE FERMENTATION OF XYLOSE BY BACTERIA OF THE AEROGENES TYPHOID GROUP CALCULATED FOR 100 C C OF CULTURE

Products	Culture 26 Gm.	B. lactis aerogenes, Gm.	B. paratyphoid B7, Gm.	B. paratyphoid B8, Gm.	B. typhoid 11, Gm.	B. typhoid 15, Gm.
Ethyl alcohol	0.4195	0.4458	0.3878	0.3070	0.1217	0.1198
Formic acid	......	......	0.0307	0.0862	0.1023	0.1045
Acetic acid	0.0513	0.0777	0.3101	0.3651	0.1590	0.1517
Butyric acid	......	......	0.0862	0.0741	0.0290	0.0166
Lactic acid	0.0156	0.0000	0.1045	0.1678	0.0000	0.0000
Succinic acid	......	......	0.6861	0.4521	0.1312	0.1171
Carbon dioxid	0.7173	0.9446	0.3051	0.4010	0.0408	0.0308
Total weight of products	1.2037	1.4681	1.9105	1.8533	0.5840	0.5405
Sugar unfermented	0.1611	0.1056	trace	trace	1.5984	—

The products obtained from the fermentation of xylose by B. paratyphoid B are formic, acetic, butyric, lactic, and succinic acids, ethyl alcohol, carbon dioxide, and hydrogen. Here the sum of the products formed are greater than those obtained from the fermen-

tation of xylose by the two strains of aerogenes. In a 2% solution of xylose these paratyphoid B organisms fermented the sugar completely and the sum of the products represents more than 92% of the original sugar. The ethyl alcohol and carbon dioxide are produced in nearly equal quantities. The two strains of paratyphoid B show a variation in the amounts of volatile acid formed; culture 8 produces somewhat larger amounts of volatile acid. Unlike the aerogenes group, these organisms form large quantities of nonvolatile acid which consist of succinic acid and lactic acid. The percentage relations of these acids are given in table 4.

TABLE 4

The Products of the Fermentation of Xylose by Bacteria of the Aerogenes - Typhoid Group Calculated for 100 C c of Culture

Products	Culture 26, Percentage	B. lactis aerogenes, Percentage	B. paratyphoid B7, Percentage	B. paratyphoid B8, Percentage	B. typhoid 11, Percentage	B. typhoid 15, Percentage
Ethyl alcohol	34.86	30.36	20.30	16.57	20.84	22.16
Formic acid	.....	.....	1.61	4.65	17.51	19.33
Acetic acid	4.26	5.29	16.23	19.70	27.23	28.07
Butyric acid	.....	.....	4.51	4.00	4.96	3.07
Lactic acid	1.29	0.00	5.47	9.05	0.00	0.00
Succinic acid	.....	.....	35.91	24.39	22.47	21.67
Carbon dioxid	59.59	64.35	15.97	21.64	6.99	5.70
Total	100.00	100.00	100.00	100.00	100.00	100.00

In the fermentation of xylose by the typhoid bacteria the products are formic, acetic, butyric, and succinic acids, ethyl alcohol, and very small amounts of carbon dioxide. The sum of the products formed, as well as the results of sugar analyses, show that these organisms utilize only a small part of the sugar, approximately 25% of the xylose in the medium. Somewhat similar results have been secured by Harden [4] for glucose; no gaseous products except carbon dioxide are formed, and this only in small amounts. In this respect the action of B. typhosus on xylose resembles that of Lactobacillus pentoaceticus. Just why the typhoid bacteria make use of such a small portion of the sugar is not known.

From a comparison of the products of fermentation obtained from xylose by the action of these different bacteria it will be clearly seen that they are widely separated as regards their biochemical properties. Although commonly discussed under the class name, colon-typhoid group, they exhibit marked differences both quantitatively and qualitatively in their action on xylose. The aerogenes forms attack this pentose sugar with the production of by-products

similar to those obtained in the alcoholic fermentation by yeasts. The paratyphoid B organisms are distinguished by their vigorous fermentation with the production of acids, particularly large amounts of succinic acid. In contrast to these two groups, the typhoid bacteria attacks xylose slowly and form only small amounts of acid, ethyl alcohol, and traces of carbon dioxide.

IDENTIFICATION OF PRODUCTS

Volatile Acid and Alcohol.—After the volatile acid from the steam distillation was titrated with 0.1 N barium hydroxide, the distillate was evaporated to dryness on the steam bath, taken up with 60-70 c c of hot water and filtered into 300 c c Erlenmeyer flasks. The acid was set free from the barium salt by the addition of the theoretical amount of 1 N sulphuric acid. The acid was added through a filter funnel drawn out to a capillary tube. After standing over night the barium sulphate was filtered and washed, and the filtrate and washings were made to 110 c c with carbon dioxide free water. This solution was then subjected to a Duclaux distillation and the distilling constants calculated from the titration data. In the case of alcohol, the acid formed by oxidation and subsequent distillation was subjected to the same manipulation. The distilling constants obtained are given in table 5. For comparison Duclaux's distilling constant for acetic acid is also given. Qualitative tests on the combined distillate and residue from the Duclaux procedure showed the presence of formic, acetic, and butyric acids. These acids were therefore present in such amounts as to give a distilling constant closely approaching that of acetic acid. For the two typhoid cultures the constants are slightly lower than for acetic acid, which indicates the presence of formic acid. The constants for alcohol run somewhat high in all cases, but as no evidence for the presence of a lower alcohol than ethyl was found, the quantity of higher alcohol present must be small.

The barium salts from the Duclaux distillation were evaporated to dryness, taken up with water and made to a volume of 100 c c. An aliquot was used for the determination of formic acid by the Fincke [11] method. The data obtained are given in table 6 and show that a large part of the volatile acid is formic acid. A strong reduction of silver nitrate also indicated the presence of formates.

[11] Biochem. Ztschr., 1913, 51, p. 253.

Another aliquot was evaporated to dryness in a platinum dish, dried at 130 C. for several hours, and the weight of the barium salts determined. These salts were ignited in the presence of an excess of sulphuric acid and the weight of barium sulphate equivalent to the organic salts was obtained. The data are given in table 7 and show that the volatile acid is a mixture of acids. The data are in general agreement with the Duclaux distilling constants and indicate the formation of some acid higher than acetic by the two paratyphoid organisms, and the formation of a lower acid by the typhoid bacteria. In view of the large amounts of formic acid found some higher acid, such as butyric, is required to give the percentages of barium sulphate and the Duclaux distilling constants obtained from the products of fermentation. A strong odor of butyric acid was easily detected when sulphuric acid was added to the barium salts before igniting them.

TABLE 5

DISTILLING CONSTANTS OF THE VOLATILE ACIDS AND ALCOHOLS OBTAINED BY THE DUCLAUX METHOD

Culture		10 C c	20 C c	30 C c	40 C c	50 C c	60 C c	70 C c	80 C c	90 C c	100 C c
Duclaux distilling constants for acetic acid	....	7.4	15.2	23.4	32.0	40.9	50.5	60.6	71.9	84.4	100
Culture 26	Alcohol	7.8	15.9	24.3	33.1	42.3	51.8	62.1	73.1	85.5	100
B. lactis aerogenes	Alcohol	7.7	15.8	24.3	33.0	42.2	51.8	62.1	73.1	85.5	100
B. paratyphoid B7	Volatile acid	7.6	15.5	23.9	32.7	41.7	51.3	61.4	72.6	85.0	100
B. paratyphoid B7	Alcohol	7.8	15.9	24.4	33.2	42.3	51.9	62.1	73.2	85.5	100
B. paratyphoid B8	Volatile acid	7.4	15.2	23.5	31.8	40.9	50.4	60.7	71.9	84.7	100
B. paratyphoid B8	Alcohol	7.8	16.0	24.4	33.2	42.4	52.1	62.3	73.4	85.7	100
B. typhoid 11	Volatile acid	7.3	14.8	23.0	30.9	39.7	49.0	59.2	70.5	83.6	100
B. typhoid 11	Alcohol	7.7	16.1	24.6	33.5	42.7	52.3	62.5	73.5	85.6	100
B. typhoid 15	Volatile acid	7.2	14.5	22.7	30.9	39.6	49.0	59.1	70.4	83.5	100

TABLE 6

THE FORMIC ACID CONTENT OF THE BARIUM SALTS OF THE VOLATILE ACIDS

Culture	Barium Salts of Volatile Acids as 0.1 N, C c	Weight of HgCl, Gm.	Formic Acid Equivalent to HgCl, Gm.	Formic Acid Calculated for 100 c c of Culture, Gm.
B. paratyphoid B7	43.5	0.1962	0.0191	0.0307
B. paratyphoid B8	36.5	0.3670	0.0358	0.0862
B. typhoid 11	24.7	0.4988	0.0487	0.1023
B. typhoid 15	37.6	0.7682	0.0749	0.1045

After due allowance is made for the formic acid, an approximate estimate of the amounts of acetic and butyric acids can be calculated from the percentage of barium sulphate. The data given in table 3 have been calculated in this way from the analytical data of tables 6 and 7.

Nonvolatile Acid.—The acids extracted by ether from the residue of the steam distillation were titrated with 0.1 N barium hydroxide after the addition of 30-40 c c of water and removal of the ether by distillation. With B. paratyphoid B 8 it was noted that crystals had separated out from the ether before the extraction flask was disconnected from the extractor. These crystals dissolved readily in water added to the ether before the titration was made.

TABLE 7

COMPOSITION OF THE BARIUM SALTS OF THE VOLATILE ACID

Culture	Barium Salts of the Volatile Acid, Gm.	Barium Sulphate Found	
		Gm.	Percentage
B. paratyphoid B7	0.5522	0.4942	89.50
B. paratyphoid B8	0.3718	0.3406	91.61
B. typhoid 11	0.3056	0.2886	94.44
B. typhoid 15	0.4314	0.4116	95.41
Theory for barium formate			102.64
Theory for barium acetate			91.37
Theory for barium butyrate			74.91

The barium salts of the nonvolatile acid were evaporated to dryness on the steam bath and then fractionated into the salts of lactic and succinic acids. For this purpose the dried salts were extracted with 10-20 c c of water, filtered into graduates, and absolute alcohol added until the concentration of alcohol was 90% by volume. A flocculent precipitate was formed and after standing in the refrigerator for a day or two, it was filtered off and washed with 90% alcohol. By this procedure the barium succinate is precipitated while the barium lactate remains dissolved in the alcohol. The greater part of the dried salts remained undissolved in the presence of a small amount of water and were later used for the determination of their barium content.

The alcohol filtrate containing the barium lactate was evaporated to a small volume, diluted with 75-100 c c water and 0.2 N zinc sulphate added in small portions until no more barium sulphate was precipated. After standing on the steam bath over night, the barium sulphate was filtered off, ignited, and weighed; from its weight the equivalent quantity of lactic acid was calculated. The barium salt remaining undissolved after the barium lactate was extracted, was transferred to a platinum dish, dried for 4 hours at 125 C. and weighed. It was then transferred to a beaker with about 100-150 c c of water. The water was heated to boiling and an excess of sulphuric

acid added to decompose the salt and to precipitate the barium as barium sulphate. In order to insure complete decomposition, the mixture of barium sulphate, undecomposed salt, and sulphuric acid was thoroughly triturated with a blunt glass rod for 10-15 minutes at the boiling point of the liquid. The beaker was left on the steam bath over night and in the morning the barium sulphate was filtered off, washed, ignited, and weighed. From the weight of barium sulphate obtained, the barium content of the unknown salt was found and proved to agree closely with that required for barium succinate. The data are given in table 8.

TABLE 8

BARIUM CONTENT OF SUCCINIC ACID PRODUCED IN THE FERMENTATION OF XYLOSE

Origin	Weight of Barium Salt, Gm.	Weight of Barium Sulphate Found, Gm.	Percentage of Barium	Percentage of of Barium in $(CH_2)_2(CO_2)_2Ba$,
B. paratyphoid B7............	1.5918	1.4472	53.6	53.8
B. paratyphoid B8............	0.5464	0.4936	53.3	53.8
B. typhoid 11................	0.1780	0.1600	53.1	53.8

The excess of sulphuric acid in the solution containing the free succinic acid was precipitated with barium hydroxide, care being taken not to add an excess of barium hydroxide. After the barium sulphate was filtered off, the solution of succinic acid was evaporated to a small volume on the steam bath and the succinic acid crystallized out by concentrating in a desiccator over sulphuric acid. A few crystals were treated with ammonia and zinc dust according to Neuberg's [12] test for succinic acid. The vapors of the fused mass produced a deep red color on a pine splinter moistened with hydrochloric acid which is characteristic for succinic acid. This qualitative test, coupled with the barium content and crystalline appearance of the free acid, clearly established the identity of the acid as succinic.

From the foregoing data, the quantity of lactic and succinic acids contained in the nonvolatile acid has been calculated and is given in table 3. In these calculations the lactic acid is found by direct determination and the succinic acid obtained by subtracting the lactic acid from the total nonvolatile acid. That this procedure leads to correct results is indicated by the data of table 8. After the barium lactate,

[12] Ztschr. f. physiol. Chem., 1901, 31, p. 574.

together with a small amount of barium succinate, was extracted, the insoluble residue of nonvolatile acid was found to be barium succinate.

SUMMARY

Xylose in yeast water peptone solutions is readily fermented by bacteria of the aerogenes and paratyphoid B groups. These organisms break up the xylose with a rapid evolution of gas. The products of fermentation with B. lactis aerogenes are essentially carbon dioxide, hydrogen, and alcohol; in this respect the aerogenes forms are somewhat similar to the yeasts. In addition to these products, small amounts of volatile acid are found. The two substances, carbon dioxide and ethyl alcohol, represent about 75% of the sugar consumed. In relation to reaction, the aerogenes organisms produce acid, at first, until the medium is about P_H 4.4; later this reaction reverts to an approximate P_H 5.0. The destruction of the sugar takes place rapidly.

The main products formed in the fermentation of xylose by paratyphoid B are formic, acetic, butyric, lactic, and succinic acids, ethyl alcohol, carbon dioxide, and hydrogen; these products represent about 92% of the original sugar. Xylose is fermented rapidly and almost completely by the paratyphoid B. organisms. In agreement with the aerogenes bacteria, these organisms form large amounts of alcohol and carbon dioxide.

The fermentation of xylose by the typhoid bacteria is far from complete. In general not more than one fourth of the xylose is decomposed. No gaseous products except small amounts of carbon dioxide were found. The chief substances are alcohol; formic, acetic, butyric, and succinic acids; and a trace of carbon dioxide. The greater part of the fermented xylose is represented by the succinic acid.

It is clearly shown from the results of this work that xylose is attacked by the organisms of the aerogenes-typhoid group with the production of volatile, nonvolatile, and gaseous substances. Although the organisms included in this study are placed in the same group, their by-products differ quantitatively and qualitatively.

THE TRANSMISSION OF SPECIFIC IMMUNE BODIES FROM THE MOTHER TO THE YOUNG

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The earlier literature relating to the transfer of antibodies from the mother to the offspring was reviewed by Morgenroth.[1] He discussed the four possibilities of the transmission of immunity: (1) the direct transmission of the newly acquired immune factor of the parents to the germ plasma (true inheritance); (2) the active immunization of mother and fetus by the same immunizing factor; (3) the passive immunity of the fetus by circulating antibodies in the mother's blood; and (4) the transmission of antibodies through the mother's milk. He concluded from the detailed experiments of Ehrlich and others that there was no actual inheritance of immunity, and that the immunity which occurred in the young of immune mothers depended partially on the intra-uterine transfer of maternal antibodies in the circulation of the fetus and partially on the transmission of antibodies through the mother's milk. In most of the earlier work on the transmission of immunity, toxins were the immunizing agents of choice, although bacteria and lysins were used to some extent. The results of the experimental work reviewed by Morgenroth were conflicting, as are those of today. Reymann,[2] for example, in his recent review of the literature on the transmission of agglutinins from mother to young, states that the majority of investigators have concluded that agglutinin was absent or less in amount in the blood of the offspring than in that of the mother, but that some investigators have found even more antibodies in the offspring's blood than in the mother's. Reymann concluded from his own experiments that the antibody content in the blood of the offspring was in some cases more and in others less than in that of the mother, and in some cases transmission by the mother's milk appeared probable. Tunnicliff,[3] in a review of the observations that had been made on the transmission of opsonins, found that the same contradictory conclusions existed as in the case of agglutinins. Tunnicliff's experiments showed that the opsonic power of serum for various bacteria was less at birth than in adult life, and that opsonins decreased during the first month of life. Eisler and Sohma[4] found that normal opsonin was transmitted from a normal mother to her offspring, but that immune opsonin was not transferred from an immunized mother to her young.

The following experiments dealt with (1) the effects of parturition on the antibody content of the serum of an immune rabbit, (2) the antibody content of the serum of the offspring of an immune rabbit, and (3) the duration of immune bodies in the serum of the offspring of immune rabbits.

Six rabbits, before pregnancy, were immunized for the antibody test. Two rabbits were immunized against red blood cells; rabbit 1 against human corpuscles and rabbit 2 against sheep corpuscles. Four rabbits were immunized against bacteria; rabbit 3 against Streptococcus viridans, rabbit 4 against type 2 pneumococcus, rabbit 5 against meningococcus, and rabbit 6 against

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[1] Kolle u. Wassermann Handbuch d. path. Mikroorg., 1904, 4, p. 784.

[2] Jour. of Immunology, 1920, 5, p. 227.

[3] Jour. Infect. Dis., 1910, 7, p. 698.

[4] Wien. klin. Wchnschr., 1908, 21, p. 684.

B. typhosus. A high degree of immunity was maintained by a weekly injection of the immunizing agent during pregnancy. Immunization was discontinued after parturition. The blood of both mother and offspring was tested for antibody content as soon after parturition as possible. It was feared that the young rabbits might not survive a bleeding from the heart, and since it was desirable to keep the offspring of each immune rabbit under observation for some weeks, only one rabbit was bled at a time until they were several weeks old. The serum from 2 rabbits and their young was examined for hemolytic amboceptor, serum from 4 rabbits and their offspring for bacterial complement fixation bodies, serum from 2 rabbits and their offspring for bacterial agglutinin, and serum from 1 rabbit and its young for opsonin.

The following technic was used for the tests: Serum inactivated by heating at 56 C. for 1 hour was used in all the tests. A normal serum was used in each test as a control for the immune serums. Hemolytic amboceptor was titrated by the usual method—to varying dilutions of inactivated immune serum 2 units of complement and a 5% homologous corpuscle suspension were added. The test was incubated at 37 C. for 2 hours, and the highest dilution of serum completely laking the corpuscles was noted. The figures given in table 1 and table 2 represent this dilution.

Complement-Fixation Antibodies.—The bacterial complement-fixation tests were made according to the original Wassermann test (one-tenth method). The antigens used in the tests were heated bacterial suspensions in normal salt solution. When there was complete inhibition of hemolysis with ¼ and ⅛ of the anticomplementary unit of antigen, the test was considered weakly positive and was indicated in the table by +. Fixation with 1/16 and 1/32 was indicated by + +, fixation with 1/64 and 1/128, by + + +, and fixation with 1/256 or over, by + + + +.

Agglutinin.—The macroscopic agglutination test was used with inactive serum and with killed bacterial suspension in normal salt solution. The mixtures were incubated at 37 C. for 2 hours and then placed in the icebox over night. The highest dilution of serum that agglutinated the bacteria was noted and was indicated by the figures recorded in tables 7 and 8.

Opsonin.—Opsonin was estimated by diluting the serum to the point of opsonic extinction, i. e., the dilution in which 50 leukocytes had the same number of cells taking part in phagocytosis as a normal control with salt solution. The points of opsonic extinction were noted as in table 9.

Hemolysin.—Antihuman hemolysin (table 1) was present in the blood of rabbit 1, 7 days before parturition in a 1 : 512 serum dilution. Immediately after the birth of the young, hemolysin was present in 1 : 256 dilution; it gradually decreased until it was 1 : 32, on the forty-first day after parturition. The blood of the young rabbit examined on the day of birth contained hemolysin in 1 : 128 dilution. As the young died on the day of birth, this was the only test made on the offspring. Antisheep hemolysin (table 2) was present in the blood of rabbit 2 in a 1 : 5,000 dilution, 1 day before it gave birth to its young. Six days after parturition its hemolysin titer was 1 : 640. It remained low until 38 days after parturition, when the rabbit developed an abscess. In a few days the titer again decreased, 1 : 80, and the rabbit died within a week. Hemolysin was present in all the young of the antisheep rabbit, but in lower titer than in the serum of the immunized mother. On the forty-fourth day all their serums contained hemolysin, and on the sixty-first day it was still demonstrable in 3 rabbits. Eighty days after birth, there was no trace of hemolysin in any of the offspring's serums. On the thirty-eight day, when the titer of the mother's serum rose to 1 : 1,280, there was no increase in the hemolysin titer of the young.

TABLE 1

Antihuman Hemolysin (Rabbit 1)

Time	Serum		
	Mother	Offspring	Normal
7 days before birth	512	...	0
Day of birth	256	128	0
2 days after birth	256	...	0
4 days after birth	256	...	0
8 days after birth	128	...	0
11 days after birth	256	...	0
16 days after birth	256	...	0
20 days after birth	128	...	0
28 days after birth	64	...	0
37 days after birth	32	...	0
41 days after birth	32	...	0

TABLE 2

Antisheep Hemolysin (Rabbit 2)

Time	Serum						
	Mother	Offspring					Normal
		1	2	3	4	5	
1 day before birth	5,000	...	...	...	...	...	0
6 days after birth	640	...	160	...	...	...	0
11 days after birth	320	160	...	...	...	...	0
16 days after birth	160	80	40	40	80	40	0
21 days after birth	320	40	40	40	80	40	0
38 days after birth	1,280	20	10	40	20	40	0
44 days after birth	80	10	5	10	5	10	0
52 days after birth	dead	2	0	2	0	2	0
61 days after birth	.....	4	5	4	0	0	0
80 days after birth	.....	0	0	0	0	0	0

TABLE 3

Complement Fixation Test with Streptococcus Viridans (Rabbit 3)

Time	Serum							
	Mother	Offspring						Normal
		1	2	3	4	5	6	
1 day before birth	++++	...	...	...	...	...	...	0
9 days after birth	0	0	...	...	...	...	...	0
11 days after birth	0	...	0	...	...	...	...	0
16 days after birth	0	...	...	0	...	...	...	0
19 days after birth	0	...	...	...	0	...	...	0
22 days after birth	+	...	...	...	...	0	...	0
30 days after birth	0	0	0	0	0	0	0	0
38 days after birth	0	+	0	0	+	+	+	0
52 days after birth	0	0	0	0	0	0	0	0
60 days after birth	0	0	0	0	0	0	0	0

TABLE 4

Complement Fixation Test with Pneumococcus (Rabbit 4)

Time	Serum		Offspring
	Mother	Normal	
1 day before birth	++++	0	Died
6 days after birth	0	0	
11 days after birth	+++	0	

TABLE 5

Complement Fixation Test with Meningococcus (Rabbit 5)

Time	Serum							
	Mother	Offspring						Normal
		1	2	3	4	5	6	
5 days before birth	++++	...	...	...	...	...	...	0
Day of birth	+	0	...	...	...	...	...	0
10 days after birth	++	...	0	...	...	...	...	0
16 days after birth	0	...	...	0	...	...	...	0
23 days after birth	+	...	...	...	0	...	...	0
33 days after birth	+	0	0	0	0	0	0	0

TABLE 6

Complement Fixation Test with B. Typhosus (Rabbit 6)

Time	Serum									
	Mother	Offspring								Normal
		1	2	3	4	5	6	7	8	
4 days before birth	++++	..	..	..	..	..	..	..	..	0
1 day after birth	++++	+++	..	..	..	..	..	..	..	0
8 days after birth	+++	..	++	..	..	..	..	..	..	0
14 days after birth	+++	..	..	+++	..	..	..	..	..	0
21 days after birth	+++	..	..	..	0	0	0	0	0	0
26 days after birth	0	0	0	0	0	0	0	0	0	0
32 days after birth	0	0	0	0	0	0	0	0	0	0

TABLE 7

Agglutinin for Meningococcus (Rabbit 5)

Time	Serum							
	Mother	Offspring						Normal
		1	2	3	4	5	6	
5 days before birth	640	...	...	...	...	...	...	0
Day of birth	40	0	...	...	...	...	...	0
10 days after birth	320	...	40	...	...	...	...	0
16 days after birth	320	...	...	320	...	...	...	0
23 days after birth	10	...	...	...	160	...	...	0
33 days after birth	160	40	10	0	10	0	0	0

TABLE 8

Agglutinin for B. Typhosus (Rabbit 6)

Time	Serum									
	Mother	Offspring								Normal
		1	2	3	4	5	6	7	8	
4 days before birth	1200	..	..	..	..	..	..	..	..	0
1 day after birth	1200	320	..	..	..	..	..	..	..	0
8 days after birth	1200	..	160	..	..	..	..	..	...	0
14 days after birth	320	..	..	160	..	..	..	..	..	0
21 days after birth	80	..	..	..	40	..	..	..	..	0
26 days after birth	80	10	20	10	80	40	10	10	10	0
32 days after birth	80	0	0	0	0	0	10	0	0	0

TABLE 9

Opsonin for B. Typhosus (Rabbit 6)

Time	Serum								
	Mother	Offspring							
		1	2	3	4	5	6	7	8
4 days before birth	640								
1 day after birth	20	20							
8 days after birth	40	..	40						
14 days after birth	40	..	..	20	20				
21 days after birth	80	..	..	..	..				
26 days after birth	40	20	20	10	10	10	20	20	20
32 days after birth	40	20	20	10	10	10	10	10	10

Complement-Fixation Antibodies.—Rabbit 3 (table 3) was immunized against Streptococcus viridans, and the serums of the immune mother and her offspring were tested for complement-fixation antibodies. The day before parturition the mother's serum gave complete inhibition of hemolysis in a streptococus fixation test. For 22 days after parturition no complement-fixing antibodies were demonstrable in either the mother's or in the offspring's blood. On the twenty-second day the mother's serum, and on the thirty-eighth day the serum of 4 of the young, slightly inhibited hemolysis. This was probably a non-specific reaction or was due to some error in technic. There was not enough serum to repeat the test.

Serum from rabbit 4 (table 4) immunized against a type 2 pneumococcus was also tested for complement-fixation antibodies. The serum gave a 4 plus fixation before the young were born and 6 days after birth a complete negative. When the rabbit's blood was tested again on the eleventh day, the complement-fixation reading was 3 plus. The young died on the day of birth.

Antimeningococcus complement-fixing antibodies were examined in the serum of rabbit 5 (table 5) and its offspring. Five days before parturition, the mother's serum gave a 4 plus fixation of complement, but on the day of birth only a 1 plus. The complement-fixation immune bodies remained low in the serum until the thirty-third day, when observation was discontinued. No demonstrable complement-fixation immune bodies were transmitted to the offspring of this rabbit.

Antityphoid complement-fixation antibodies were tested in rabbit 6 (table 6) and its young. Both before and after parturition the mother's serum gave complete inhibition of hemolysis in the typhoid complement-fixation test. The

titer for complement-fixation immune bodies remained high for 3 weeks, but on the twenty-sixth day it dropped to zero and remained there. Serum from the offspring of this rabbit had a high complement-fixing antibody content for 14 days after birth, but this entirely disappeared during the third week.

Agglutinin.—The agglutinin titer (table 7) was tested in the serum of the antimeningococcus rabbit (table 7) and in the serum of its offspring. There was a drop from 640 to 40 in the antimeningococcus rabbit serum after it gave birth to its young. The agglutinin content of the mother rabbit's serum fluctuated. On the thirty-third day, the last time it was examined, the titer was 1 : 160. The young rabbit examined on the day of birth had no demonstrable antimeningococcus agglutinin. On the sixteenth day the agglutinin titer was the same for the immunized rabbit and its young. On the thirty-third day 3 of the young rabbits had antimeningococcus agglutinin in their blood. The agglutinin content (table 8) of the serum of the antityphoid rabbit and its young was also examined. The agglutinin titer of the mother was high, 1 : 1,200, and remained at this point for 8 days after parturition. After this time it decreased rapidly, and on the twenty-first day it was only 1 : 80, at which point it remained until the thirty-second day, when the last examination was made. The serum of 1 of the offspring, examined a day after birth, contained antityphoid agglutinin in 1 : 320 serum dilution. All serums from the young contained agglutinin on the twenty-sixth day but on the thirty-second day only 1 serum contained agglutinin.

The serum of the antityphoid immune rabbit was examined for opsonin (table 9). Four days before parturition 1 : 640 serum dilution was the point of opsonic extinction, and 1 day after parturition the point of opsonic extinction had dropped to 1 : 20. The opsonic content of the serum remained low as long as the rabbit was under observation. All the serums of the offspring contained opsonin (usually lower in quantity than in the mother), which persisted the 32 days in which the rabbits were observed.

SUMMARY

Antisheep and antihuman hemolysin was decreased in immunized rabbits after parturtition. The serum of their offspring contained hemolysin, but in lesser amounts than in the immune mother. There was little hemolysin in the serum of the young at the end of the sixth week and none at the end of the eleventh week.

Complement-fixing antibodies almost disappeared from the serum of 3 of the immune mothers (3, 4, 5) after parturition: In rabbit 3 there was no return of these immune bodies; in rabbit 5, only a slight increase of complement-fixing antibody; in rabbit 4, a return of the complement-fixing antibody. Rabbit 6 did not have an immediate loss of complement-fixing antibodies after parturition, but had marked decrease of these antibodies a week later. Complement-fixing antibodies could be determined in the offspring of rabbit 6 only, and were not present in these young rabbits after 2 weeks.

Agglutinin decreased in the serum of rabbit 5 and remained constant in rabbit 6 after parturition. Agglutinin was present in the

serum of the offspring (one exception—table 7) but in lesser amount than in the mother. The young of both immune mothers had a low agglutinin titer at the end of the fourth week.

The point of opsonic extinction in rabbit 6 fell from 640 to 20 after parturition. Opsonin remained low. The serums of the offspring had low points of opsonic extinction, but opsonin persisted after 5 weeks.

CONCLUSIONS

There is considerable variation in the antibody content in the serum of rabbits. The agglutinin and complement fixing antibodies appeared stable in the serum of rabbit 6 when they were compared with the immune bodies in other rabbits. Complement-fixing antibodies appear to be less stable than the other immune bodies studied and less readily transmitted to the young.

The results of these experiments do not indicate whether the young receive a passive immunity from the immune mother or whether they receive antibodies from the mother's milk. After parturition there was a marked decrease in the antibody content of the serum of the immunized rabbits, and this suggests a possible cause for the many post-partum infections. The offspring of immune rabbits, as a rule, have antibodies in their serum which persist in appreciable but decreasing amounts for 4 to 6 weeks.

BLOOD CHANGES AND ANTIBODY PRODUCTION IN HUMAN BEINGS AFTER INJECTION OF PNEUMOCOCCUS LIPOVACCINE *

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Epidemics of acute respiratory diseases occurring during the last few years have caused many attempts to immunize against the organisms isolated in these infections, with the hope that results comparable to the immunization against B. typhosus might be obtained. Among the various vaccines tried a pneumococcus lipovaccine was used extensively in some of the army camps and to a lesser extent among civilians.

In March, 1919, for the purpose of experiment, I inoculated 3 healthy men with the army pneumococcus lipovaccine. One c c of this vaccine contained 0.83 mgm. of the following: types 1, 2 and 3 dried pneumococcal protein. The dose was 1 c c injected subcutaneously. All 3 men had moderately severe local reactions and general constitutional symptoms, consisting of slight rise in temperature, headache, backache, muscular pain, and general malaise. Blood counts were taken on each person at 2 to 3 day intervals for several weeks, then at longer intervals, No. 1 for 3 months, No. 2 for 6 months, and No. 3 for 1 year. At the same time, blood was taken aseptically from the median vein of each person. The serum was collected from the blood, inactivated by heating at 56 C. for 30 minutes, and examined for agglutinin for each pneumococcus type. Selected serums were tested for complement-fixing antibodies and for protective bodies. The pneumococci used for the test were types 1, 2 and 3, which were furnished by the Rockefeller Institute for Medical Research.

BLOOD

There was no apparent change in the blood picture that could be attributed to the vaccine. In No. 1 (table 1) the leukocytic count dropped from 16,000 to 14,200; in No. 2 (table 2) it rose from 7,650 to 9,450; and in No. 3 (table 3) it remained constant, about 10,000.

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In all three cases, after the injection of the vaccine, there was an increase in the percentage of polymorphonuclear leukocytes and of the eosinophiles, the latter rising from 3% to 10% in No. 1.

AGGLUTININ

The macroscopic agglutination test was used with different dilutions of inactive serum and a constant amount of an 18-hour culture. The mixtures were incubated for 2 hours at 37 C., then placed in the icebox over night before the readings were noted. Several normal serums and a known positive serum as controls were set up with each test.

Before vaccination, serum from No. 1 and No. 2 contained no demonstrable agglutinins for any of the 3 types of pneumococci. Serum from No. 3 in a 1:4 dilution agglutinated type 3 pneumococcus.

TABLE 1

J. A. H.—Findings After Vaccine Injection March 14, 1919

Date	Blood Count							Agglutinin for Pneumococcus			Complement Fixation
	White Blood Cells	Red Blood Cells	Differential					Type 1	Type 2	Type 3	
			P., %	L., %	E., %	B., %	L. M., %				
3/14/19	16,000	4,000,000	68	22	3	1	5	0	0	0	0
3/17/19	14,200	4,400,000	79	16	0	1	4	0	0	2	
3/19/19	12,000	4,200,000	57	31	10	0	2	0	0	4	
3/21/19	12,000	4,200,000	63	33	2	0	2	4	2	S	0
3/24/19	7,000	4,000,000	62	34	3	0	1	4	2	8	
3/25/19	6,400	4,800,000	75	21	2	0	2	512	512	32	+
4/ 1/19	8,100	3,910,000	69	25	2	0	6	512	512	64	
4/ 3/19	6,650	3,100,000	68	20	4	3	4	512	512	256	+++
4/ 7/19	8,250	4,160,000	69	22	1	0	8	512	512	512	
4/18/19	6,250	4,990,000	65	28	0	0	6	512	512	512	+++
4/24/19	9,100	4,900,000	56	40	1	0	3	256	512	256	
4/30/19	8,700	5,100,000	66	31	1	0	2	40	40	40	
5/14/19	9,850	5,120,000	63	28	5	0	4	128	128	128	
5/23/19	7,250	4,000,000	65	32	0	1	2	10,000	1,280	25,000	++++
6/ 4/19	8,650	4,420,000	67	31	1	0	1	10,000	1,280	5,000	
6/30/19	7,750	3,770,000	70	22	1	0	7	10,000	1,280	5,000	++++

P. = Polymorphonuclear leukocytes
L. = Lymphocytes
E. = Eosinophiles
B. = Basophiles
L. M. = Large mononuclears
+ = Slight inhibition of hemolysis
+++ = Partial inhibition of hemolysis
++++ = Complete inhibition of hemolysis

Figures under agglutinin indicate the highest dilution of serum agglutinating.

Serum 1 (table 1) 7 days after vaccination, in 1:4 dilution, agglutinated pneumococcus type 1; in 1:2 dilution, pneumococcus type 2 and in 1:8 dilution, pneumococus type 3. Normal serum, however, in these dilutions occasionally agglutinated pneumococci, so that it was not until the eleventh day that agglutinins were unmistakably

increased above the normal. At this time a 1:512 serum dilution agglutinated types 1 and 2, and 1:32 agglutinated type 3. Types 1 and 2 remained almost constant for a month, and agglutinin for type 3 gradually increased to 1:512. On the forty-fourth day, there was a sudden drop to 1:40 for all 3 types. There was no constitutional disturbance that coincided with this agglutinin decrease, and there was only a slight change in the blood picture. The next observation was made 2 weeks later, when all types were agglutinated by 1:128. The height of agglutinin production, 1:10,000 for type 1, 1:1,280 for type 2, and 1:25,000 for type 3, was reached in another week, 69 days after inoculation. The titer was still high when the final observation, 106 days after vaccination, was taken. At that time agglutinin for type 1 was demonstrable in 1:10,000 serum dilution, for type 2 in 1:1,280 dilution, and for type 3 in 1:1,280 dilution.

TABLE 2

W. J.—FINDINGS AFTER VACCINE INJECTION MARCH 14, 1919

Date	Blood Count							Agglutinin for Pneumococcus			Complement Fixation
	White Blood Cells	Red Blood Cells	Differential					Type 1	Type 2	Type 3	
			P., %	L., %	E., %	B., %	L. M., %				
3/14/19	7,750	4,200,000	61	38	0	0	1	1	1	1	0
3/16/19	9,450	4,950,000	70	27	0	0	3	2	1	2	
3/18/19	6,900	4,200,000	76	23	2	0	0	2	2	2	
3/20/19	5,300	3,990,000	55	40	1	0	4	16	2	4	+
3/22/19	6,350	4,150,000	69	26	2	1	2	16	2	4	
3/24/19	6,050	4,450,000	58	41	1	0	0	16	2	4	
3/26/19	9,400	4,000,000	68	28	0	0	4	640	320	160	
3/28/19	6,700	4,590,000	64	28	3	0	5	1,280	320	320	+++
3/30/19	8,300	4,380,000	70	27	1	1	1	640	320	320	
4/ 2/19	6,850	4,820,000	52	43	2	0	3	640	640	320	++
4/ 9/19	6,300	4,450,000	60	33	4	0	3	20	1,280	1,280	++++
4/15/19	8,850	4,420,000	39	57	2	0	2	1,280	1,280	2,560	
4/25/19	5,700	4,640,000	50	44	1	1	4	640	1,280	1,280	
5/ 9/19	10,600	4,810,000	68	29	0	0	3	10	1,280	1,280	++++
6/ 4/19	8,400	4,800,000	61	33	1	0	5	640	2,560	160	
8/28/19	9,200	4,900,000	65	34	0	1	0	320	1,280	320	++++

P. = Polymorphonuclear leukocytes
L. = Lymphocytes
E. = Eosinophiles
B. = Basophiles
L. M. = Large mononuclears

\+ = Slight inhibition of hemolysis
++ = Partial inhibition of hemolysis
+++ = Partial inhibition of hemolysis
++++ = Complete inhibition of hemolysis

Figures under agglutinin indicate the highest dilution of serum agglutinating.

Serum 2 (table 2) agglutinated in low dilutions for 10 days after inoculation. On the twelfth day the agglutinin titer suddenly rose to 1:640, 1:320, 1:160 for pneumococcus types 1, 2 and 3 respectively. The height of agglutinin production occurred 31 days after vaccination, when the readings for all three types were all 1:1,280. When

the last observation was made, 5½ months after inoculation, agglutinin was present in 1 : 320 serum dilution for types 1 and 3 and in 1 : 1,280 for type 2. On the twenty-sixth and fifty-sixth days there was a drop in agglutinin for type 1. No clinical cause was demonstrable for this drop, though on the fifty-sixth day the leukocyte count was higher than normal for this individual.

TABLE 3

J. H.—Findings After Vaccine Injection March 14, 1919

Date	Blood Count							Agglutinin for Pneumococcus			Complement Fixation
	White Blood Cells	Red Blood Cells	Differential					Type 1	Type 2	Type 3	
			P., %	L., %	E., %	B., %	L. M., %				
3/14/19	10,200	4,800,000	74	19	1	1	5	0	0	4	0
3/16/19	10,500	6,000,000	77	16	6	0	1	32	0	2	
3/18/19	10,200	5,600,000	58	30	6	1	5	16	0	8	
3/21/19	8,400	4,900,000	68	27	5	0	0	32	0	64	0
3/23/19	7,700	4,600,000	74	20	[illegible]	0	6	16	0	64	
3/25/19	8,100	4,800,000	73	21	3	2	1	200	64	400	
3/27/19	10,450	4,940,000	71	24	3	0	2	32,768	64,000	32,768	++
3/29/19	10,650	4,500,000	69	22	2	0	7	32,768	128,000	64,000	
4/1/19	9,900	4,760,000	75	18	3	0	4	32,768	512	512	++
4/4/19	7,650	4,700,000	68	28	4	0	0	640	1,280	640	
4/9/19	7,600	4,000,000	68	28	2	0	2	640	2,520	640	
4/15/19	7,950	4,380,000	56	40	4	0	0	5,040	5,040	5,040	
4/23/19	8,350	4,180,000	63	34	3	0	0	640	1,280	2,520	
4/30/19	8,000	4,500,000	65	32	1	0	1	320	640	640	++++
5/6/19	8,200	4,780,000	67	30	1	0	2	1,280	1,280	640	
5/13/19	11,800	4,200,000	63	33	2	1	1	320	5,040	2,500	
5/23/19	9,600	4,300,000	60	39	1	0	0	2,500	5,400	20.000	++++
6/13/19	8,560	5,000,000	62	42	0	1	5	5,400	5,400	20,000	
6/27/19	7,800	4,080,000	60	33	2	1	4	5,400	5,400	10,000	++++
7/15/19	7,500	5,100,000	75	20	0	0	5	10,800	5,400	10,000	
10/3/19	8,200	5,100,000	60	28	7	1	4	5,400	5,400	10,000	
11/12/19	10,400	3,400,000	67	20	2	0	11	64	64	64	
2/26/20	8,500	4,900,000	65	28	2	1	4	64	64	32	++

P. = Polymorphonuclear leukocytes
L. = Lymphocytes
E. = Eosinophiles
B. = Basophiles
L. M. = Large mononuclears
\+ = Slight inhibition of hemolysis
++ = Partial inhibition of hemolysis
++++ = Complete inhibition of hemolysis

Figures under agglutinin indicate the highest dilution of serum agglutinating.

Serum 3 (table 3) contained agglutinin for type 3 in a 1 : 4 dilution before the man received the pneumococcus lipovaccine. Agglutinins for types 1 and 3 were produced at once, and agglutinins for type 2 after 11 days. In 13 days the titer for all types was unusually high, and in 15 days the highest point was reached. On this date, types 1, 2 and 3 were agglutinated by the following serum dilutions: 1 : 32,000, 1 : 28,000 and 1 : 64,000. There were variations in agglutinin content, but the level remained high for 7 months. The serum in the seventh month agglutinated types 1 and 2 in 1 : 5,400 dilution and type 3 in 1 : 10,000 dilution. The next observation made 6 weeks

later showed no agglutination for any pneumococcus type above a 1 : 64 serum dilution. The final test with this serum was made 11½ months after vaccination. At this time 1 : 64 serum dilution agglutinated pneumococcus types 1 and 2 and 1 : 32 serum dilution, type 3.

Six persons who had received the pneumococcus lipovaccine a year previously had their blood tested for pneumococcus agglutinins. The results are tabulated in table 4. All serums had agglutinin for pneumococcus type 1, and serums 1, 4 and 6 for types 2 and 3. Serum 3 was taken from a patient convalescing from a typical case of influenza, uncomplicated by pneumonia.

COMPLEMENT FIXATION TESTS

A number of serums from each man were examined for complement-fixing immune bodies. Two antigens were used throughout the tests. The first antigen was the army pneumococcus lipovaccine emulsified in alcohol and further diluted in normal salt solution. The second antigen was a suspension in normal salt solution of heated washed pneumococci—types 1, 2 and 3 in equal amounts—obtained from 18-hour broth cultures. The technic was that of the original Wassermann test (one-tenth method), and has been previously described.[1] The readings with the two antigens were practically the same.

Serum taken before the 3 men were vaccinated had no demonstrable complement-fixing bodies. The first fixation of complement occurred in serum 1 in 14 days, in serum 2 in 7 days, and in serum 3 in 15 days. On the twenty-fifth day after inoculation, the 3 serums gave 4 plus fixations. Serums 1 and 2 were still 4 plus when observations were discontinued, 3 and 6 months after vaccination. Serum 3 gave a 4 plus fixation until the ninth month, and at the end of 11½ months, a 2 plus fixation.

PROTECTIVE EXPERIMENTS

Selected serums were used in protective experiments on white mice. The tests were made according to the method described by Cole and his associates.[2]

Great difficulty was experienced in increasing the virulence of the pneumococci used in the test and in maintaining that virulence after it was obtained. Broth cultures from the heart blood, and from the

[1] Howell, K., Jour. Infect. Dis., 1918, 22, p. 230.

[2] Acute Lobar Pneumonia, Monograph of the Rockefeller Institute, 1917, No. 7.

peritoneal fluid, and peritoneal washings were passed through series of mice. Frequently, when the organism had apparently gained a high degree of virulence, this virulence would suddenly drop, and the succeeding mice fail to die. Several times, for example, when the heart blood was plated, to make sure of the purity of the strain before using it in the protective tests, and when colonies were transferred to broth, all within a period of 24 hours, there was such decrease in the virulence of the organism that further mouse passage was necessary before protective tests could be made.

TABLE 4

INDIVIDUALS OTHER THAN THOSE REPRESENTED IN TABLES 1, 2 AND 3 WHO HAD RECEIVED PNEUMOCOCCUS LIPOVACCINE ONE YEAR PREVIOUS TO TESTING

Cases	Agglutinin for Pneumococcus		
	Type 1	Type 2	Type 3
1	250	32	128
2	500	8	0
3	500	0	0
4	500	128	64
5	1,000	0	0
6	1,000	16	16

Figures indicate the highest dilution of serum agglutinating.

In the final tests, the virulence of the strains was not high. The lethal dose of pneumococcus type 1 was 0.00005, of pneumococcus type 2, 0.00001, and of pneumococcus type 3, 0.001. Serums in 0.2 c c amount, before the men were vaccinated and for 9 days after vaccination did not protect mice. Serums for the remaining protective experiments were chosen when the agglutinin titer was high. As a rule, the control set of mice died within 24 hours, and the set of mice with serum in 48 to 72 hours.

DISCUSSION

Among the reports that have been made within the last 2 years on the immunity reactions after prophylactic pneumococcus vaccine are the following: Whitmore, Fennel, and Peterson [3] inoculated 2 men with a pneumococcus lipovaccine containing types 1 and 2. One man had agglutinin for type 1 in a 1 : 25 serum dilution, which was still existant after 6 weeks. The serum (1 : 20 dilution) agglutinated type 2 for 2 weeks. A second man's serum agglutinated type 1 in 1 : 15 dilution and type 2 in 1 : 40 dilution. No agglutinins were present in

[3] Jour. Am. Med. Assn., 1918, 70, p. 427.

this serum after 6 weeks. Fennel [4] found that 10 weeks after injection with the pneumococcus lipovaccine 0.2 c c of serum of an inoculated person protected a mouse against a fatal dose of pneumococci. Cecil and Austin [5] demonstrated agglutinin in 27 of 42 individuals 8 days after vaccination with the pneumococcus lipovaccine and found their serum afforded a definite degree of protection to white mice. Whitmore found that agglutinin was present in the serum of vaccinated men after the eighth day and that the immunity curve rose steadily after that time and continued a considerable period. Blake and Cecil [7] vaccinated a series of monkeys with a pneumococcus lipovaccine, and their serums tested 2 weeks after injection failed to show demonstrable agglutinin and protective bodies. In later experiments, Cecil and Blake [8] by subcutaneously injecting living virulent pneumococcus type 1 produced some agglutinin response when the blood was tested after 2 weeks. Serum from these monkeys taken 20 days after vaccination protected white mice against 1,000 times the minimal lethal dose of pneumococcus type 1. Cecil and Blake in their series of experiments found that sometimes when immune bodies were not demonstrable, monkeys vaccinated with pneumococcus type 1 were still protected against pneumonia of the homologous type and even at times against the other fixed types of pneumonia. They conclude from their experiments that active immunity against pneumonia was largely independent of the presence or absence of agglutinins and protective bodies in the serum of monkeys. Hitchens [9] also decided that immunity did not always coincide with the presence of immune bodies in the serum after pneumococcus vaccination.

The protective results of pneumococcus vaccine were noted at two large army camps. For example, at Camp Custer, Cecil and Austin [10] found that among the 12,519 vaccinated men there were no cases of pneumonia of the 3 fixed types during the 10 weeks after vaccination, while among the 20,000 unvaccinated men there were 26 cases during the same period. At Camp Wheeler Cecil and Vaughan [11] gave prophylactic pneumococcus lipovaccines to 13,460 men, 80%

[4] Jour. Am. Med. Assn., 1918, 71, p. 2115.
[5] Jour. Exper. Med., 1918, 28, p. 19.
[6] Harvey Lecture, Jan. 11, 1919.
[7] Jour. Exper. Med., 1920, 31, p. 519.
[8] Jour. Exper. Med., 1920, 31, p. 657 and p. 685.
[9] Med. Rec., 1919, 96, p. 692.
[10] Jour. Exper. Med., 1918, 28, p. 19.
[11] Jour. Exper. Med., 1919, 29, p. 457.

of the force. Excluding the cases developing in the first week, only 8 cases of pneumonia produced by the fixed type occurred.

In the case of the few individuals, doctors and nurses, of the Michael Reese Hospital, who were injected with a pneumococcus lipovaccine, no pneumonia developed and in only one case influenza, uncomplicated by pneumonia, developed during the year in which they were under observation. It would appear that immune bodies after lipopneumococcus vaccine develop slowly, not reaching their height for a considerable time, and persist for a duration of at least several months. It would appear therefore that agglutinin might have been demonstrated in many more of the reported cases, if serum had been tested for agglutinin later than the 8 day or 2 week period that most of the observers chose.

SUMMARY

Blood: After the pneumococcus lipovaccine there was a slight rise in the polymorphonuclear leukocytes and in the eosinophiles.

Agglutinin: There was a definite rise in agglutinin in the 3 serums examined in 11 to 12 days. The height of agglutinin titer was reached on the 25th, 31st and 15th days, respectively. The agglutinin titer remained high for No. 1 for 3 months (last observation); for No. 2 for 6 months (last observation); for No. 3 for 9 months. Demonstrable agglutinin was present in 7 persons for at least a year.

Complement-Fixing Immune Bodies: Complement-fixing antibodies were present in the serum of the 3 men examined on the 14th, 7th, and 15th days, respectively. The height was reached in 25 days, and all 3 serums showed some degree of fixation in the last test made on each serum.

Protective Experiments: Protective bodies for pneumococcus types 1, 2 and 3 were demonstrable in some degree in the 3 serums at the time the agglutinin titer was high.

There was no apparent correlation between the clinical history, the blood count, and the antibody content.

CONCLUSION

Agglutinins, complement-fixing bodies, and protective bodies for pneumococci types 1, 2 and 3 are demonstrable in the serum of individuals vaccinated with pneumococcus lipovaccine. These antibodies appear in the serum rather late and are present to some degree for at least one year.

THE PRODUCTION BY STREPTOCOCCUS HEMOLYTICUS OF AN AGGLUTININ FOR RED CORPUSCLES WHICH INHIBITS HEMOLYSIS

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An apparently typical hemolytic streptococcus was isolated from a vegetative growth on an aortic valve at necropsy. Cultures had been made from the ground vegetative growth on human blood-agar plates; and in 36 hours there appeared small, elevated, white, moist colonies, which were surrounded by a clear zone of hemolysis, 2 to 3 mm. in diameter. Films from a colony, stained by Gram's method, showed a small round gram-positive coccus occurring in pairs and in short chains. The coccus was not soluble in bile, nor did it ferment inulin. Classified according to its sugar reactions (Andrews and Horder classification), it belonged to the Streptococcus pyogenes group. The virulence of this streptococcus was tested on white mice, and 0.06 c c of an 18-hour broth culture was found to be the lethal dose.

The ability of this streptococcus to lake blood was further tested by adding 0.5 c c of a 5% sheep corpuscle suspension to each of a series of 10 tubes. Each tube contained 0.5 c c of culture dilutions of an 18-hour broth culture, the amounts being graduated as follows: the first tube, 0.5 c c of culture; the second tube, 0.25 c c, the third tube, 0.125 c c, etc. The tubes were examined after 2 hours' incubation at 37 C., and no hemolysis had occurred. When the tubes were shaken, the corpuscles were found to be agglutinated, in some tubes forming a solid mass which was impossible to break up by shaking the mixture. The tests were repeated under the same and under different conditions. A series of tubes containing uninoculated broth was set up thereafter as a control. At no time were the corpuscles in the plain broth control tubes agglutinated. The following modifications were made in the test: whole defibrinated sheep blood was used instead of the washed corpuscles; human corpuscles, rabbit corpuscles, mouse corpuscles and guinea-pig corpuscles were substituted for the sheep corpuscles; the quantity of 18-hour broth culture was kept a constant, and the amount of blood varied; 24, 48 and 72-hour broth cultures were used instead of the 18-hour broth culture; the incubation period was lengthened. In all of the tests there was marked agglutination.

There was some hemolysis when the tests were incubated for 12 hours, a degree of hemolysis that corresponded with that in the broth control tubes.

An attempt was made to estimate roughly the amount of agglutinin in an 18-hour broth culture by the following method: A tube containing 0.5 c c of the streptococcus broth culture and 0.5 c c of 5% human corpuscle suspension was incubated 1 hour, then centrifugated, and the supernatant removed from the agglutinated corpuscles and incubated with fresh red blood corpuscles for one hour; the fresh corpuscles were agglutinated, and after centrifugation the supernatant fluid agglutinated the third addition of fresh human corpuscles.

In order to determine whether the agglutinin had modified the red blood cells so that they could not readily lake, a fragility test and a hemolytic test were made on the human corpuscles agglutinated by the streptococus broth culture and on fresh human corpuscles. The degree of hemolysis in the tests on the treated corpuscles and in the tests on the fresh corpuscles was practically the same in each instance.

The effect of heat on the agglutinin was tested. Streptococus broth cultures of 5 days' and 1 day's growth were heated at 60 C. for 1 hour. Human corpuscles were added to each tube, and the tubes were incubated at 37 C. for 2 hours. The corpuscles were agglutinated but not hemolyzed. The tests were repeated with cultures heated at 70 C. for one hour and with cultures heated at 80 C. for one hour. The corpuscles were still agglutinated, and not hemolyzed.

Eighteen-hour streptococcus broth cultures were centrifugated for 2 hours. The supernatant fluid was removed. The streptococci were washed 3 times in sterile normal salt solution and suspended in sufficient normal salt to make a faintly cloudy suspension. Series of 10 tubes containing dilutions (details given under broth culture test) of the supernatant fluid (table 1) and washed corpuscles, human, sheep, guinea-pig, rabbit, and mouse, were incubated at 37 C. for 2 hours and placed in the icebox over night before final readings were taken. The same procedure (table 2) was followed with the suspension of washed streptococci. The supernatant fluid in 1:512 dilution agglutinated all the different species of corpuscles. Undiluted, it slightly laked as well as agglutinated the corpuscles. The streptococcus suspension did not act uniformly with the different species of corpuscles; it had no effect on guinea-pig corpuscles, it partially agglutinated sheep corpuscles, and in low dilutions it laked human, rabbit, and mouse corpuscles, while in high dilutions it agglutinated them.

The hemolytic tests were repeated after one month. Corpuscles were partially agglutinated, and partially laked. The streptococcus strain was passed through a series of white mice to discover whether the agglutinin that was decreasing could be increased. The strain had little virulence, 1 c c of the culture failing to kill a mouse. Neither the virulence nor the power to agglutinate increased after mouse passage.

TABLE 1

SUPERNATANT FLUID OF STREPTOCOCCUS CULTURES

Dilutions	Human Corpuscles		Sheep Corpuscles		Guinea-Pig Corpuscles		Rabbit Corpuscles		Mouse Corpuscles	
	H	A	H	A	H	A	H	A	H	A
1:2	+	+	+	+	+	++	+	+++	+	++
1:4	0	++	+	+	0	++	0	+++	+	++
1:8	0	++	+	+	0	++	0	+++	+	++
1:16	0	++	+	+	0	++	0	+++	+	++
1:32	0	++	+	+	0	++	0	+++	+	++
1:64	0	++	+	+	0	++	0	+++	+	++
1:128	0	++	+	+	0	++	0	+++	+	++
1:256	0	++	+	+	0	++	0	++	+	++
1:512	0	++	+	+	0	++	0	++	+	++

\+ = Slight
++ = Partial
+++ = Complete

H = Hemolysis
A = Agglutination

TABLE 2

WASHED STREPTOCOCCUS SUSPENSION

Dilutions	Human Corpuscles		Sheep Corpuscles		Guinea-Pig Corpuscles		Rabbit Corpuscles		Mouse Corpuscles	
	H	A	H	A	H	A	H	A	H	A
0	+++	0	0	++	+	+	+++	0	+++	0
1:2	+++	0	0	++	0	0	+++	0	+++	0
1:4	+++	0	0	++	0	0	+++	0	+++	0
1:8	+++	0	0	++	0	0	+++	0	+++	0
1:16	++	0	0	++	0	0	+++	0	++	++
1:32	++	0	0	++	0	0	+++	0	+	++
1:64	+	0	0	++	0	0	++	++	+	++
1:128	0	+	0	++	0	0	0	++	+	++
1:256	0	+	0	++	0	0	0	++	0	++
1:512	0	+	0	++	0	0	0	++	0	++

\+ = Slight
++ = Partial
+++ = Complete

H = Hemolysis
A = Agglutination

Tested after 4 months, the entire broth culture and the supernatant fluid laked corpuscles in 1 : 4 dilutions. The streptococcus suspension caused no hemolysis. Corpuscles were not agglutinated by the broth cultures, by the filtrate, or by the streptococcus suspension. At this time the strain was not virulent for white mice, rabbits or guinea-pigs

SUMMARY

The streptococus here considered appeared to be a typical Streptococcus hemolyticus when it was cultivated on blood-agar medium, but it had the peculiar quality of agglutinating instead of laking red blood cells when it was grown in broth medium. It seems possible that, on the blood-agar cultures, agglutination of the corpuscles was prevented by the mechanical factor of the solid medium and that accordingly hemolysis took place as usual. The tests noted suggest that the agglutinin for red blood corpuscles was an exogenous product of the bacterial cell and that hemolysin was probably present, but that its action on corpuscles, for some reason not determined, was inhibited by the agglutinin present. Agglutinin production was a transient quality of this streptococcus strain, since it was present in an appreciable amount for only 6 weeks.

A COMPARISON OF ANTIGENS FOR BACTERIAL COMPLEMENT FIXATION *

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Bacterial complement fixation is one of the methods used at present in testing for immune bodies in serum. The success of the test seems to depend largely on the use of a reliable bacterial antigen. It has been our custom to use a simple antigen, merely a heated washed suspension of bacteria in normal salt solution. Since other antigens have been described by other writers as being preferable, we decided to compare, in a few bacterial complement-fixation tests, this antigen with antigens prepared by other methods, as to specificity, anticomplementary and antigenic properties, and antigenic range. The following antigens were used for the tests:

Antigen 1.—This was an autolysate antigen, originally used by Blake [1] for precipitin tests. Washed bacteria were dried in vacuo and suspended in normal salt solution in the proportion of 10 mg. per c c. This suspension was repeatedly frozen and thawed until a faintly opalescent fluid, free from bacterial bodies, resulted. Dilutions of this opalescent fluid in normal salt were used as antigen.

Antigen 2.—Antigen 2 was prepared according to the method used by Kinsella and Swift.[2] Washed sediment from 24-hour cultures was suspended in 5 c c each of normal salt solution and of absolute alcohol. The precipitate formed was centrifugalized, desiccated in vacuo, ground and weighed. To 10 mg. of the resulting precipitate, 5 c c of 2% antiformin was added, and the suspension placed in the water bath at 56 C. until the bacteria were dissolved. The solution was neutralized with 0.1 N sulphuric acid (litmus indicator), and 1 to 2 drops of 5% sodium thiosulphate were added to free the chlorin, the end results being tested with potassium iodid starch paper. The solution was made up to 10 c c with carbolized normal salt solution and centrifugalized. One c c of this antigen equaled 1 mg. of the dried ground precipitate.

[1] Jour. Exper. Med., 1917, 26, p. 67.
[2] Jour. Exper. Med., 1917, 25, p. 877.

Antigen 3.—The third antigen was prepared according to a method described by Krumwiede and Noble,[3] a method especially favored by them as a rapid means for the production of a good precipitin antigen. Sufficient antiformin was added to a heavy suspension of bacteria in distilled water, to make a final concentration of 5%. This suspension was boiled for several minutes for the purpose of dissolving the bacteria. When they were dissolved, there was an appearance of translucency. If this appearance was not obtained, the process was repeated until the bacteria were in solution. The solution was neutralized with N/1 hydrochloric acid. Several volumes of 95% alcohol were added, the suspension was centrifugalized, and the supernatant fluid was decanted and used as antigen.

Antigen 4.—Warden [4] considered that specific fats rather than proteins might be the antigenic factor in bacterial complement-fixation antigens and prepared some with that in view, which proved successful. His method of extracting fats was not used by us, but Noguchi's [5] method of extracting a lipoid constituent. Bacterial cultures were washed off with normal salt solution, centrifugalized, and the bacterial sediment dried in vacuo. The dried bacteria were extracted in absolute alcohol. The alcoholic extract was evaporated to dryness, dissolved in ether, and precipitated by acetone. The precipitate was weighed, and 0.3 gm. was dissolved in 1 c c of ether and 9 c c of methyl alcohol. This solution was the stock antigen. For the complement-fixation tests one part of the stock antigen was added to 9 parts of normal salt solution.

Antigen 5.—Antigen 5 was prepared according to the method employed by Olitsky and Bernstein.[6] Bacteria were suspended in distilled water, and the suspension was allowed to autolyze at 60 C. for 1 hour and then at 37 C. for 24 hours. The suspension was then centrifugalized until a clear supernatant fluid resulted. This fluid was normalized with 0.9% salt solution and used as antigen.

Antigen 6.—In the preparattion of antigen 6, Gay's modification of Besredka's method was employed.[7] Cultures grown on solid medium were washed off with normal salt solution. The salt suspension was precipitated with an equal quantity of absolute alcohol.

[3] Jour. Immunology, 1918, 3, p. 1.
[4] Jour. Infect. Dis., 1918, 22, p. 133.
[5] Serum Diagnosis of Syphilis, p. 79.
[6] Jour. Infect. Dis., 1916, 19, p. 253.
[7] Kolmer, Infection, Immunity, and Specific Therapy, p. 474.

and the mixture centrifugalized. The sediment was dried in vacuo, weighed, and ground into a fine powder with sufficient crystals of salt to make a 2% suspension of dried material in an isotonic salt solution, when a proper amount of water was added. This suspension, not filtered or centrifugalized, was diluted with normal salt solution and constituted the antigen.

Antigen 7.—Antigen 7 was an autolysate prepared by a method given by Kolmer.[7] Cultures grown on solid medium were washed off with normal salt solution. The emulsion was centrifugalized thoroughly, and the resulting sediment dried over sulphuric acid. The dried material was ground with crystals of salt, and sufficient distilled water was added to render the solution isotonic. Each c c of the suspension contained about 0.05 gm. of the dried material. The emulsion was then shaken for 24 hours and centrifugalized, and the supernatant (preserved with 0.5% alcohol) used as antigen.

Antigen 8.—Antigen 8 was the heated bacterial suspension in normal salt, mentioned previously as our usual bacterial antigen. Bacteria were washed 3 times in normal salt solution, suspended in normal salt solution, and heated at 56 C. for 1 hour. Heating was for the purpose of killing, not autolyzing, the bacteria.

Although it is generally accepted that the bacterial antigenic property lies either in the bacterial body itself or in the endotoxic substances that are liberated when the bacterial cells are autolyzed, nevertheless entire bróth cultures of the 4 bacteria used in the tests were used as antigen and also supernatant (decanted after 2 hours' centrifugalization at high speed) from these cultures. An uninoculated broth was used as a control. The latter had no antigenic property. The supernatant and entire cultures had a wide antigenic range and excellent antigenic properities but both were nonspecific, giving about as strong fixations with other immune serums and even normal serums as with the homologous serums. Both were, therefore, discarded as antigens.

The 8 antigens were made for each of the following bacteria: pneumococcus, streptococcus, B. typhosus and meningococcus.

Rabbits were immunized against the same bacterial strains that were used for the antigens. Each antigen was tested for its anticomplementary unit, for its antigenic unit with the homologous immune serum, for its specificity, and for the antigenic range.

The complement-fixation tests were made by the method previously described by us.[8] The proportions were those used in the original Wassermann test, in $\frac{1}{10}$ quantities. The first incubation period was 1 hour at 37 C., and the second incubation period about 30 minutes at 37 C.

RESULTS

Antigen 1: Blake's autolysate, obtained by repeated freezing and thawing, was anticomplementary in the following amounts: pneumococcus antigen, 0.2 c c; streptococcus antigen 0.5 c c of a 1 : 10 dilution; typhoid antigen, 0.5 c c; meningococcus antigen 0.4 c c. It was strongly antigenic (table) for antistreptococcus and antimeningococcus serums, but only weakly binding for antipneumococcus and antityphoid serums. The antigenic range for the latter 2 antigens was therefore rather limited. Control serums were slightly fixed with the antistreptococcus and antityphoid antigens. Judged by the results of these tests, the meningococus and streptococcus antigens prepared by this method were excellent, but the pneumococcus and typhoid antigens poor.

Antigen 2: The autolysate produced by antiformin were anticomplementary in the following amounts: pneumococcus, 0.5 c c; streptococcus, 0.05 c c of 1 : 100 dilution; typhoid, 0.05 c c of 1 : 1,000 dilution, and meningococcus, 0.05 c c of undiluted antigen. There was no antigenic unit for the hemologous antipneumococcus serum (table), a very low antigenic unit for antityphoid and antimeningococcus serums, and a high antigenic unit (1/1024 of the anticomplementary unit) and consequently a wide antigenic range, for the antistreptococcus serum. This method was considered to produce a good antigen for streptococcus only and was not entirely satisfactory here, since it gave slight fixation of complement with a heterologous serum.

Antigen 3, prepared by the rapid antiformin method, was anticomplementary in the following amounts; pneumococcus, 0.05 c c; streptococcus, 0.05 c c; typhoid, 0.05 c c; meningococcus, 0.2 c c. Its antigenic property was good, and the antigenic range was wide. It gave nonspecific fixation in $\frac{1}{4}$ of the anticomplementary unit in the case of the streptococcus antigen, but the antigenic range here was so great that it was still considered a satisfactory antigen.

Antigen 4, the lipoid antigen, was anticomplementary for pneumococcus in 0.4 c c, for streptococcus in 0.05 c c, for typhoid in 0.1 c c,

[8] Howell and Anderson, Jour. Infect. Dis., 1919, 25, p. 1.

and for meningococcus in 0.05 c c amounts. The antigenic unit was good in the case of the pneumococcus and streptococcus antigens, being 1/512 of the anticomplementary unit, while in the case of the typhoid and meningococcus antigens it was only 1/8 of the anticomplementary unit and consequently a weak antigen. There was also nonspecific fixation with the typhoid antigen which, together with the low antigenic unit and slight antigenic range, would disqualify it as an antigen for typhoid complement-fixation tests.

Antigen 5, autolyzed by heat, was anticomplementary in the following amounts: pneumococcus, 0.4 c c; streptococcus, typhoid and meningococcus, 0.05 c c. Its binding unit was good in the case of pneumococcus and streptococcus and fair in the case of typhoid and meningococcus. The antigens, however, were rather nonspecific in action and on this account not entirely satisfactory.

TABLE 1

COMPLEMENT FIXATION WITH DIFFERENT ANTIGENS

Serums	Antigens							
	1	2	3	4	5	6	7	8
Antipneumococcus	8	0	32	512	512	64	16	16
Normal control	0	0	0	0	8	0	0	2
Antimeningococcus control	0	0	0	0	0	0	0	0
Antistreptococcus	2068	1024	2068	512	256	32	512	4136
Normal control	2	0	4	0	4	4	0	0
Antityphoid control	0	2	4	0	4	4	0	0
Antityphoid	8	2	16	8	16	2	64	64
Normal control	2	0	0	2	2	0	2	4
Antistreptococcus control	2	0	0	2	0	0	2	4
Antimeningococcus	512	16	32	8	16	32	16	128
Normal control	0	0	0	0	0	0	0	0

Numerals = fraction of anticomplementary unit of antigen that gave complete inhibition of hemolysis, i. e., 8 = 1/8 of anticomplementary unit.

Antigen 6, the alcoholic precipitate suspended in normal salt solution, was anticomplementary in the following quantities: pneumococcus, 0.5 c c; streptococcus, 0.1 c c of 1 : 100 dilution; typhoid. 0.1 c c; meningococcus, 0.05 c c. The antigenic unit was 1/64 of the anticomplementary unit for pneumococcus and 1/32 of the anticomplementary unit for streptococcus and meningococcus. There was practically no binding power for typhoid, 1/2 of the anticomplementary unit. The streptococcus antigen was nonspecific in its action.

Antigen 7, the filtrate from organisms dried, ground, and shaken for 24 hours, was anticomplementary in the following amounts: pneu-

mococcus, 0.05 c c; streptococcus, 0.1 c c; typhoid, 0.1 c c; meningococcus, 0.05 c c. The antigenic unit of the pneumococcus and meningococcus antigens was $\frac{1}{16}$ of the anticomplementary unit, of the streptococcus antigen $\frac{1}{512}$, and of the typhoid antigen $\frac{1}{64}$. There was slight nonspecificity of action with the typhoid antigen only.

Antigen 8, the bacterial suspension, was anticomplementary in the following amounts: pneumococcus, 0.4 c c; streptococcus, 0.05 c c; typhoid, 0.1 c c of 1 : 10 dilution; meningococcus, 0.4 c.c The antigenic units were: pneumococcus, $\frac{1}{16}$ of the anticomplementary unit; streptococcus, $\frac{1}{4136}$ of the anticomplementary unit; typhoid, $\frac{1}{64}$ of the anticomplementary unit; meningococcus, $\frac{1}{128}$ of the anticomplementary unit. Pneumococcus was nonspecific in $\frac{1}{2}$ of the anticomplementary unit and typhoid in $\frac{1}{4}$ of the anticomplementary unit, which lessened the antigenic range for these antigens.

Since these antigen tests were completed, an article has appeared by Matsuwoto[9] on a comparison of the methods of preparing B. typhosus antigen. In the 9 antigens that he used, he found a 14-day broth culture, killed by heating at 60 C. for one hour, to be most antigenic. The antigen tested by us that corresponded most nearly to this was the entire broth culture discarded in spite of its high antigenic quality because of its nonspecificity. The antigen second in antigenic value was a suspension of living bacteria in normal salt solution. This corresponded closely to our suspension of heated bacteria in normal salt solution, which was found to be the most satisfactory antigen for our typhoid bacterial complement-fixation tests.

SUMMARY

In summing up the antigens (table), it appears that the bacterial suspension was at least as satisfactory as any other one antigen. It was not anticomplementary in small quantities; the antigenic unit was better than that of the other antigens for streptococus and typhoid; and it ranked second for meningococcus and ranked fourth for pneumococcus. The antigenic range was good, since even in the pneumococcus test there was complete fixation in 3 dilutions. It, like the other antigens, in low dilutions of the anticomplementary unit, gave nonspecific reactions with certain serums, but the antigenic range was wide enough so that there was marked difference between these reac-

[9] Jour. of Immunology, 1920, 5, p. 111.

tions and fixation with homologous immune serum. Antigen 1 was the most satisfactory of the antigen preparations for the meningococcus test, and antigens 4 and 5 for the pneumococcus test. Since there is such diversity in the choice of the most satisfactory bacterial antigens for these 4 bacteria, and since the bacterial antigenic factor has not been determined, it would seem advisable before starting an extended bacterial complement-fixation study, as a preliminary, to determine the antigen best suited for the bacteria used in that test.

PRACTICAL METHODS IN THE PURIFICATION OF OBLIGATE ANAEROBES

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Nearly every one agrees that many of the descriptive data in the older publications on obligative anaerobes were vitiated by impurity in the cultures examined. While care was usually exercised that no aerobic forms were present, no doubt many cultures have been described as single species in cases where two or more obligate anaerobes were associated. This fact has been responsible for much of the past and some of the present confusion in the literature on the taxonomy of this group.

Whatever method is used to limit a bacterial culture to anaerobes, there is no justification for the asumption of purity of species therein. Until reasonable assurance can be given that a culture does not contain two or more species, differential tests are of no value farther than to show the possible presence or absence of certain organisms with outstanding peculiarities. Thus the production of tetanus in susceptible animals by injection of a toxic filtrate or culture, or of botulism by feeding, may indicate the presence of these organisms but does not prove the purity of the culture. Where special organisms are being looked for, such tests should be made with enriched cultures previous to attempted isolation; but the folly of recording metabolic properties of cultures not consciously and carefully purified is obvious.

It is needless to dwell on this point at length; most of the recent investigators have recognized the necessity of certain purity in anaerobic as well as in aerobic cultures.[1] As Fildes[2] has stated, "the whole problem of anaerobic growth centers around the isolation of pure cultures." The great majority of "new" methods for the culti-

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[1] Meyer, Jour. Infect. Dis., 1915, 17, p. 458. McIntosh and Fildes, Lancet, 1916, 1, p. 768. Henry, Brit. Med. Jour., 1917, 1, p. 763 and p. 806. Donaldson, Lancet, 1917, 1, p. 82. Wilson and Steer, Brit. Med. Jour., 1918, 2, p. 568. Stoddard Jour. Am. Med. Assn., 1918, 70, p. 906. Weinberg and Seguin, La gangrène gazeuse, 1918; Hemple, Jour. of Hyg., 1918, 17, p. 13.

[2] Med. Research Com., Spec. Report Series 12, 1917.

vation of obligative anaerobes have had the goal of surface cultures with a view to facilitating isolation.

There is still a great deal of divergence of opinion as to the best method of securing isolated cultures of unquestioned purity. Thus Weinberg and Seguin,[1] than whom none has been more successful in the study of obligative anaerobes, have used the deep culture method throughout as a means of isolation; the English investigators, McIntosh and Fildes,[1] Henry,[1] Wilson and Steer,[1] Adamson,[3] and the members of the Medical Research Committee led by Bullock,[4] have used surface culture methods. In this country, Meyer,[1] Dickson and Burke[5] used the deep culture method successfully, while Stoddard[6] has followed the English in the use of surface methods. In addition to the above, there have been scattering attempts to utilize purely microscopic means of isolation.

This paper summarizes certain observations in the isolation of a considerable number of cultures among which have been identified B. sporogenes, 18 strains; B. welchii, 3 strains; B. tetani, 1 strain; B. botulinus, 3 strains; B. chauvaei, 1 strain; vibrion septique, 7 strains; and several others not yet completely identified.

INITIAL CULTURE AND ENRICHMENT

The usual occurrence of mixed flora of aerobes and anaerobes in nature makes some method of enrichment of the latter generally desirable as a preliminary step to complete elimination of the aerobes and isolation of the anaerobes, especially in those cases in which growth may have come to a standstill, as in canned goods, or in soil or water. Where active pullulation is in progress, one may proceed at once to dilution and isolation. Thus McIntosh[7] advocated the direct streaking of pus from war wounds, when possible, as giving a more nearly correct estimate of the proportion of various organisms originally present. But the usual occurrence of facultative anaerobes is likely to make actual isolation a complicated problem.

The alternate plan of enrichment previous to isolation permits a choice of methods from a considerable variety of procedures. The

[3] Jour. Path. and Bacteriol., 1919, 22, p. 345.
[4] Med. Research Com., Special Report Series 39, 1919.
[5] Jour. Am. Med. Assn., 1918, 71, p. 518.
[6] Lancet, 1919, 196, p. 12; Jour. Exp. Med., 1919, 29, p. 187.
[7] Med. Research Com., Special Report Series 12, 1917.

constricted tube with marble seal[8] using glucose broth (P_H 7-8) as a medium is preferable for this purpose from the standpoints of simplicity and efficiency.

Inoculation should be immediately preceded by heating the medium for a few minutes in a bath of boiling water and cooling to 50 C. The material under examination should be introduced partly below the seal and partly above.

The temperature of incubation should be 37 C. usually; even B. botulinus, which, according to most of the older authors, grows better at 20-25 C., in our experience grows more rapidly and heavier at 37 C. and produces strong toxin at that temperature also.

Growth of anaerobes in the constricted tube naturally starts below the seal. But as soon as the evolution of gas begins even cultures of anaerobes free from aerobes are likely to produce turbidity above the seal as well as below due to the fact that each bubble of gas, whether it be carbon dioxide, hydrogen, methane, hydrogen sulphide, or a mixture of these and others, acts as a vacuum so far as other gases, including oxygen, are concerned. Each escaping bubble carries its load of oxygen, until the medium becomes anaerobic throughout. This explanation accounts satisfactorily also for the ease with which merely deep tubes of medium may be made to yield anaerobic growth, especially if previously boiled out, or aerophilic organisms be present, or a nidus for the initiation of growth in the depths be present. Late growth near or even on the surface does not therefore necessarily imply the presence of aerobic organisms.

Twenty-four hours' incubation are generally sufficient, but in some cases 48 are necessary, and complete sterility cannot be assured within a week at least. In making a sterility test of biologic products such as serums and vaccines, which, incidentally, is the specific purpose for which the constricted tube was invented, an incubation period of one week is recommended by the Hygienic Laboratory of the United States Public Health Service. In certain instances in which the bacterial cells have been subjected to injury, as, for example, in the heating of bacterial vaccines, concentration of tuberculin, or canning processes (Burke,[9] 1919), the period of bacterial lag may be prolonged considerably.

Closely second to the constricted tube for enrichment of anaerobes are deep brain and meat mediums. Introduced by Von Hibler,[10] these

[8] Univ. Calif. Pub. in Path., 1915, 2, p. 147.

[9] Jour. Bacteriol., 1919, 4, p. 555.

[10] Ueber die pathogenen Anaeroben, 1908.

mediums were first used extensively in this country by Meyer[1] and during the war they were employed widely in work with anaerobes. Bradley[11] has shown the utility of brain medium in preserving the viability of the meningococcus and Holman[12] has emphasized the value of a cooked meat medium in special and routine work. While the general results with these mediums are similar, as shown in the records of the English writers,[3,4] who have used meat largely, while the French have used mostly brain, the consistency and whiteness of the brain medium predispose one to its preference. As usually prepared, it consists merely of a mixture of ground sheep or beef brains with water, equal parts, tubed to a depth of 10-12 cm. in culture tubes. Sheep brains are preferable as being less bloody, since it is not customary to stun sheep by a blow on the head before killing; otherwise there is little or no difference as to source. There is a distinct advantage in the addition of a modicum of glucose to stimulate rapid growth and gas formation, but an excess is to be avoided as prohibiting sporulation (generally) and interfering with the blackening of the medium, which is characteristic of the putrefactive anaerobes and therefore of diagnostic value. An addition of peptone is also advantageous in stimulating early development and intensifying the pigmentation mentioned above. The following is the present method of preparation:

1. Boil sheep brains with an equal volume of distilled water.
2. Decant water (save) and press brains through a potato ricer.
3. Add decanted water, 2% peptone, and 0.1% dextrose.
4. Tube by punching through the filling funnel with a glass rod, filling tubes about half full.
5. Sterilize intermittently in the Arnold sterilizer. Five daily runs of 30 minutes each are recommended.

Sterilization in the autoclave has usually been a failure owing to the tendency of the medium to splatter up onto the plugs. It should be and remain nearly white with a clear supernatant liquid above the brain substance. Growth is indicated by turbidity and in many cases by production of gas. The presence of glucose provides for those organisms, such as the tetanus bacillus, which appear to sporulate better in its presence, but the small amount used is exhausted shortly so that those organisms which would be prevented from sporulation

[11] Jour. Am. Med. Assn., 1918, 70, p. 1816.
[12] Jour. Bacteriol., 1919, 4, p. 149.

by an excess retain their vitality for considerable periods of time. Brain medium therefore serves for storage as well as for enrichment.

Milk favoring the development of B. welchii has been a favorite medium for enrichment of material from war wounds. Defensible in that case, since the principal other pathogenic anaerobes in war wounds, excepting B. tetani, but including the vibrion septique and B. oedematiens, are lactose fermenters, the general use of milk might exclude, or at least suppress, certain nonlactose fermenting microorganisms.

English writers have developed the idea of selective enrichment quite fully and have shown that the predominating flora of a mixed meat culture, for example, varies from day to day in a regular order, when the material has come from a badly infected wound. There are 3 periods, each marked by certain metabolic and morphologic characteristics of the predominating organisms. At the end of the first 24 hours, organisms of the fermentative type, such as B. welchi, B. fallax, and vibrion septique predominate; in another day putrefactive organisms, such as B. sporogenes, are in the ascendency, while after some days of further incubation, nonfermentative bacteria without marked putrefactive properties such as B. tetani appear. It happens that the spores of the organisms of the first two periods are central or subterminal while those of the last are terminal, so that the metabolic characteristics of the changing flora are correlated with distinctive morphologic properties.

These facts have found their application in the provision of carbohydrate mediums for the enrichment of fermentative bacteria, and of sugar-free protein rich mediums for putrefactive organisms.

Some of the English scientists have advocated also the use of animals for enrichment. When a single pathogenic anaerobe is known to be associated with nonpathogenic species the latter can often be eliminated by animal passage through normal animals susceptible to the first. For a mixture of pathogenic organisms it is stated that actively or passively immunized guinea-pigs or mice can be used as a means of selective elimination.

The writer has had little occasion to test the use of animals in this manner; some attempts to eliminate the hay bacillus from a culture of B. welchii were less successful than the use of inhibitive dyes shortly to be described. At any rate the use of selective mediums and

selective animals should not be thought of as means of isolation; the English writers have made it clear that these are only preparatory to plating; they are, in other words, merely enrichment methods.

ELIMINATION OF AEROBES

Here again the question might be raised as to the propriety of postponing the isolation process. Ideally desirable, it is practically difficult to secure pure cultures of anaerobes from cultures mixed with aerobes previous to the employment of certain procedures for elimination, or at least suppressing the latter. After one has secured the primary culture one may begin at this point the separation of the anaerobic from the aerobic examination. If the latter is important, suitable solid medium, e. g., blood-agar plates or slants in the case of pathologic materials, should be streaked out for the isolation of the aerobes; otherwise, a plain agar slant is usually sufficient to indicate the possible presence of aerobes and their general nature, as shown by incubation at 37 C. and subsequent Gram stain. In case no growth occurs aerobically on solid mediums the culture can be assumed to be free from aerobes. Such a test for the exclusion of aerobic contamination should be made frequently in anaerobic work and in every case in which important conclusions are to be drawn as a criterion of aerobic contamination.

Two general categories of methods are available for the elimination of aerobes from cultures containing anaerobes, namely, selective heating and selective cultivation in special mediums.

Selective heating is applicable to sporulating anaerobes only in the presence of nonsporulating contaminants. This limitation necessitates the use for most anaerobes of culture medium having less than an excess of fermentable carbohydrate, as, for example, brain medium, or the alkaline egg medium of Robertson,[13] in order to insure sporulation if possible. A temperature of 85 C. for 10-15 minutes is usually sufficient to destroy all vegetative forms; occasionally a higher temperature or longer exposure may be required. To avoid loss of the initial culture subcultures should be taken before as well as after heating.

When aerobic spores are present, selective heating is usually a failure, since most of the anaerobic spores are less resistant, with the possible exception of B. sporogenes. For the elimination or suppression of these, selective culture methods may be utilized.

[13] Jour. Path. and Bacteriol., 1916, 20. p. 237.

One of these consists in the rapid growth and frequently repeated transfer of cultures of saccharolytic organisms, such as B. welchii in glucose broth as advocated by Taylor [14] and Tullock.[15] Tullock has applied the same idea in the selective cultivation of nonfermentative organisms such as B. tetani in medium fermented out with B. sporogenes.

The use of certain tri-amino-tri-phenyl-methane dyes, such as gentian violet and crystal violet, for the elimination of hay bacilli from cultures of obligative anaerobes,[16] is also recommended. The following is a transcript of a characteristic record showing how this method is operated:

B. sporogenes No. 66 contaminated with aerobic spores, 2/26/20 Glucose broth G.V.

```
                                                                            {Glucose broth G.V.  gas
                                                    {Glucose broth G.V.  gas{Deep meat.......   —gas
                                {Glucose broth G.V. {Deep meat.......++gas  {Plain agar.. —  —  —
    {Glucose broth G.V.  gas    {Deep brain..........gas{Plain agar.........  ++
Gas {Deep brain........+gas     {Plain agar aerobic.. +
    {Plain agar aerobic  +
```

Culture now considered aerobe free.

The method can nearly always be made to work sufficiently to eliminate completely all the aerobic spores, but if it cannot, there is a great advantage in using it as a preliminary suppressive measure immediately preceding isolation.

In all selective methods there is the possibility of losing desirable organisms from the culture, and one should take the precaution of holding stock cultures of the initial culture until it is certain that none of the organisms it contains has been missed.

PURIFICATION

Purification may be accomplished frequently from original material, even that containing aerobes, but if the attempt is not successful immediately some of the anaerobe enrichment and aerobe suppression procedures just described should be carried out.

SURFACE METHODS OF ISOLATION OF ANAEROBES

The writer was engaged intermittently during five years previous to 1917 in testing various of the described methods, with indifferent

[14] Jour. Path. and Bacteriol., 1916, 20, p. 384.
[15] Med. Res. Com., Special Report Series 39, 1919.
[16] Jour. Am. Med. Assn., 1919, 72, p. 61.

success, until he was led to the following modification of Wright's [17] method: Rabbit and horse blood agar slants prepared from the usual meat infusion nutrient broth with 3% agar plus 5-10% sterile defibrinated blood have been used mostly. The tubes should be plugged with absorbent cotton. The slanted surface should be moderately dry at the time of inoculation although a small amount of water of condensation in the lower angle of the slant does no harm if precautions be taken to prevent its flowing over the inoculated surface on inversion of the tubes. Inoculation may be from any suitable liquid culture; we have used mostly glucose broth, constricted tube cultures or the supernatant fluid portion of deep brain cultures, avoiding, in the latter case, the transfer of solid particles. The slants are streaked by beginning at the bottom, diluting in the water of condensation (or hysteresis) if any, and spreading over the surface of the slant by slowly withdrawing the loop, at the same time making rapid transverse movements over the agar. Replacing the cotton plug in the usual manner it is then cut off squarely with the scissors and pushed into the tube so that its top is about 1.5 cm. below the mouth of the tube, and it does not touch any part of the slanted medium. The space above the cotton plug is then filled with pyrogallic acid crystals (0.5-1.5 gm.) and a rubber stopper selected of such size as to plug the opening snugly. With everything ready, the tube is then held in the left hand with the inoculated surface toward the right; 1-2 c c of 10% lye are poured on the crystals from a bottle and the rubber stopper placed in position. The tube must be inverted at once and without allowing the moisture in the tube to flow over the inoculated surface, that is, it must be made to flow down into the stopper over the glass surface of the tube in order to prevent spreading colonies. The obvious purpose of inverting the tubes is to avoid soiling the culture with the lye-pyrogallol solution.

Incubation at 37 C. usually gives growth within 24-48 hours; yet certain cultures of B. putrificus from Professor Sturgis of Yale University rarely showed evidence of growth in less than 5 days, at which time one could rely fairly regularly on finding an abundant culture. This interesting fact might be interpreted as indicating a somewhat lower oxygen requirement on the part of this organism, which was met in this method only by a somewhat longer prolonged operation of the oxygen absorbing mixture.

[17] Jour. Bost. Soc. Med. Sci., 1900, 5, p. 114; also Centralbl. f. Bakteriol., 1. 1901, 29, p. 61.

Satisfactory surface growth can be secured in approximately nine trials out of ten. Well separated colonies are a matter of experience in dilution as they would be under aerobic conditions and have been obtained in about three fourths of the trials. Attempts to secure possible latent nongrowing bacilli from the bare spaces between well separated colonies were never successful. Isolated colonies can be picked into deep brain medium or glucose broth in constricted tubes with impartial success; only occasionally does a picked culture fail to grow.

With this method some 40 cultures now under study in this laboratory were purified. The details will be discussed in connection with the description of the cultures. Reliance was never placed in a single streaking and picking for isolation, but the purification has been repeated in each instance at least 3 times and in some cases 8 times before purity was finally assumed. Meticulous care to detect the persistence of impurity or the appearance of a new contamination was exercised constantly. This procedure seems as reliable as the repeated streaking and picking of aerobes under analogous conditions and justifies the opinion that the isolation of these cultures was thoroughly complete.

But the finding of two cultures, one of B. welchii and one of B. tetani, apparently contaminated anaerobically after they were supposedly purified as above, served to point out a possible fallacy in all methods of surface culture isolation of obligative anaerobes. This conviction was supported by the observation of a culture known to be impure in which one organism grew for a time in a very narrow range of oxygen tension only, as indicated in deep glucose agar. The method of isolation from deep glucose shake cultures will be discussed shortly in order to indicate some possible theoretical limitations in all cultural methods of isolation, but which apply especially to surface culture methods. However, evidence is lacking to show that the considerations to be named are of anything more than theoretical importance; at any rate, they must appear only rarely. The contamination found in the tetanus and Welch bacillus cultures mentioned above was not attributable to any fault of isolation since earlier duplicate cultures in both cases made subsequent to isolation failed to show the impurity, which was probably due to contaminated medium. Furthermore, a rigid examination of the remaining cultures since isolation has failed to show a single instance of anaerobic contamination attributable to faulty isolation.

DEEP CULTURE METHODS OF ISOLATION

One of the oldest methods of isolating anaerobes and at the same time one of the best, if not actually the very best, the deep culture method of Hesse,[18] has been more widely used, perhaps, than any other cultural means of anaerobe cultivation and isolation. It utilizes ordinary culture tubes, or better still, the rubber stoppered tubes of Burri,[19] which may be filled to a depth of 10-12 cm. with either agar or gelatin medium. The writer has used mostly 1% agar in meat infusion broth adjusted automatically to a constant alkalinity by means of magnesium carbonate in excess which is allowed to settle out of suspension before the agar cools, by holding in the Arnold sterilizer for an hour or two at 100 C. After cooling, the container, usually a 1,000 c c conical graduate, is inverted on a clean surface to eject the solid mass and that portion containing the sedimented excess of magnesium carbonate and precipitate is cut off, leaving the remainder clear and with a nearly constant reaction of P_H equal approximately to 8.1, which is quite suitable for the growth of most sporulating anaerobes. Excellent gelatin can be made in the same manner. It has been customary to add 0.2% to 1.0% glucose to the agar, to stimulate growth, at this point. Glucose should not be heated along with the other ingredients in the presence of magnesium carbonate, owing to excessive hydrolysis which is likely to occur. If excessive splitting of the medium occurs during growth of the organisms, the reduction of the glucose content to a minimum goes far to avoid the difficulty and is preferable to the use of potassium nitrate as advocated by Veillon and Maze.[20]

With young brain or glucose broth seed cultures one can usually secure suitable dilutions to provide well separated colonies by rinsing the inoculated loop in a tube of sterile water. It has seemed unnecessary to shake mixed cultures with sand as advocated by Stoddard,[1] though it is conceivable that circumstances might arise in which this would be desirable. Ordinarily the sporulating anaerobes develop in glucose broth or in the supernatant fluid of brain mediums in a homogeneous suspension; the individual bacteria fall apart readily and do not tend to form conglomerate masses which would be likely to contain more than one species. Inoculation should be made in boiled

[18] Deutsch. med. Wchnschr., 1885, 11, p. 214.
[19] Centralbl. f. Bakteriol., II, O., 1902, 8, p. 533.
[20] Comptes. rend. Soc. biol., 1910, 68 p. 112.

mediums cooled down to 40-50 C., care being taken to distribute the organisms well by rotation of the inoculated tube previous to solidification.

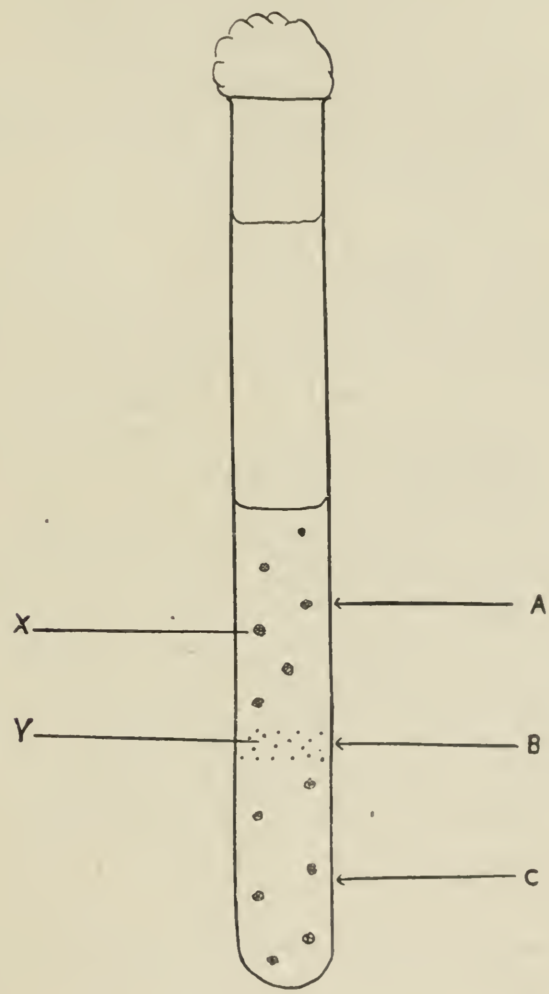
Most cultures yield growth within 24-48 hours' incubation at 37 C. If well separated colonies appear, the tube can be cracked near the base with a file and encircled with a hot rod or electric wire to complete the fracture, or, in the case of Burri's tube, the rubber stopper is removed, and the agar plug is pushed out from the other end into a sterile dish. It is now sliced transversely into disks 4-6 mm. thick, with a scalpel boiled in 1-2% lye, thus exposing to view the colonies embedded in the medium. The intervening agar is partly seared away from such of these as it is desired to cultivate, as advocated by Burke,[21] and the selected colony removed en masse with a slender picking needle to deep brain, glucose broth, or other suitable medium. The searing of the agar is to be considered an important part of this method as avoiding the transfer of organisms other than those inclosed in the colony itself. This procedure, especially if several times repeated, gives results equal to those obtained by surface culture methods.

During the use of the deep culture method an observation was made which would have been overlooked in Wright's method or any other surface culture method, and as such would constitute a serious drawback to the use of surface culture methods for isolation, if it occurred frequently, which is doubtful.

A certain culture, later shown to contain B. sporogenes, on diluting in glucose agar, presented, after incubation, two distinct types of colony, one an obligative anaerobe "Y" growing only in narrow range (about 5 mm.) at a constant depth below the surface, i. e., 3 cm.; the other a facultative anaerobe "X," developing throughout the depth of the medium. No colonies of "X" were ever observed to invade the range of "Y." The situation is diagramed roughly in the illustration. Several points arise: First, "X" colonies picked into deep brain always developed as a mixture with "Y"-like organisms, owing doubtless to latent "Y" organisms lying in the picked colony. It was easy to determine the admixture because "X" proved to consist of Staphylococcus albus, while "Y" consisted of rods. In case "X" colonies had ever been found lying within the range of "Y," each might have been secured free from the other; there was no difficulty in picking "Y"

[21] Jour. Bacteriol., 1919, 4, p. 555.

free from "X" the first time. As it turned out, "X" proved to be aerobic and only facultatively anaerobic so that "Y" could be eliminated easily by repeated surface culture; in case "X" had been an obligative anaerobe there would have been more difficulty in securing a pure culture. A similar instance of an organism with a narrow range of oxygen tension tolerance occurred in the case of an obligatively anaerobic micrococcus recovered from the nasal washings of a patient with acute rhinitis.[22]



A tube of deep glucose agar containing two types of colony; one grew in a narrow range of reduced oxygen tension, the other grew throughout the tube.

If one examines the possibilities of such a culture in Wright's method, he may note that the oxygen tension is reduced probably in a given instance to a definite, but unfortunately, unknown point

[22] Hall, Unpublished manuscript.

at which a balance occurs with the absorbing mixture of alkaline-pyrogallol. This point probably varies from time to time according to the strength of reagents used, size of air space, etc. Suppose the oxygen tension reduction corresponds to a point "A" or "C" in the deep agar; no colonies of "Y" would appear. If it should happen to strike a point analogous to "B" and conditions are similar to those that seemed to exist in the deep tube, no colonies of "X" would appear, though the reason for their failure to appear in the range of "Y" in either case is somewhat unclear, unless it be a question of antagonism, for which there is no direct evidence.

These somewhat theoretical considerations led to a reconsideration of microscopic methods of isolating single organisms as means of avoiding the above mentioned difficulties.

MICROSCOPIC METHODS OF ISOLATION

The preparation of pure cultures in general has been reviewed carefully by Jörgensen,[23] who has pointed out the value of direct optical observation of the development of a single cell completely isolated, a method quite feasible with yeasts and molds but much less so with bacteria on account of their small size.

As early as 1821, Ehrenberg followed the germination of certain fungi microscopically, as did also Mitscherlich in 1843, Kützing in 1851, and Schülze in 1860, that of yeast cells, and Tulasne in 1861, and De Bary in 1866, that of bacterial spores. In 1881, Brefeld invented the moist chamber for cultivation of organisms under microscopic observation which was especially emphasized by Hansen as a means of purifying yeasts. Braatz[24] devised, in 1890, a special slide for the cultivation of anaerobes over alakline-pyrogallol in hanging drops, and the hanging drop method of isolation was further developed by Lindner in 1893. The method consists in a preliminary dilution, in drops of sterile medium hanging from the under side of a sterile cover, such that each drop contains approximately one cell only; those drops which meet this expectation, as shown by microscopic examination, are marked and transferred, after incubation, as pure cultures. A modification aiming to facilitate the detection of organisms in such drops was the use of India ink, as suggested by Burri.

[23] Micro Organisms and Fermentation, 1911.

[24] Centralbl. f. Bakteriol., 1890, 8, p. 520.

A modern development of microscopic methods of isolation is found in the technic of Barber,[25] which involves the use of a capillary pipet in picking single organisms out of a diluted suspension under the microscope. Donaldson [1] appreciated the theoretical advantages of optical isolation of anaerobes, but his recognition of the technical obstacles led him to the advocacy of a method of dilution involving a hemocytometer count followed by culture of amounts theoretically such as to distribute the organisms singly in separate tubes—virtually a return to the old dilution methods of Lister and Hansen in use prior to the invention of solid mediums by Koch.

The cultivation of aerobic bacteria from single cells has been carefully investigated recently by Hort,[26] who refers to six distinct methods. Hort considers the optical difficulties and the use of liquid medium in these methods insurmountable obstacles to the continuous observation from single cell to colony which he considers necessary. His own method of covering thinly spread dilutions on agar slabs with cover slips for observation might be successful for anaerobes, but this has not been claimed.

The writer's efforts have been restricted to Barber's method. After seeing Professor Barber isolate a pair of pneumococci, one cannot doubt the general efficacy of the procedure in the hands of so skilful a master of technic. My own persistent attempts have never met with anything but the most dismal failure in the use of his method, but Hempl [1] has claimed to use Barber's method quite successfully.

In conclusion, the method of culture in deep glucose agar seems quite satisfactory, especially if repeated isolation is practiced with each culture. Actually, one usually secures a pure culture the first time.

SUMMARY

This article calls attention to the relatively recent appreciation of the failure of some early investigators to secure unquestionably pure cultures of obligative anaerobes.

The methods of initial culture and selective enrichment are discussed and certain observations are noted on the elimination of aerobic contaminants through selective heating and selective cultivation.

[25] Jour. Infect. Dis., 1911, 8, p. 349; Phil. Jour. Sc., 1914, B9, p. 307.
[26] Jour. Hygiene, 1920, 18, p. 307.

Certain limitations on cultural methods of isolation, especially surface methods, are suggested, and a critical review of the present status of surface culture methods, deep culture methods and microscopic methods of isolation, is given.

It is concluded that the method of isolation by deep cultivation in glucose agar is most practical.

THE TESTING OF GERMICIDAL SUBSTANCES AGAINST THE GONOCOCCUS

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In gonorrhea, a disease second to none in frequency and economic and social importance, the treatment is almost entirely empiric. The literature contains but few experimental studies on this subject, and the few to be found are incomplete and inconclusive. In undertaking a series of laboratory researches on venereal disease, we have attempted, by experimentation, to lay down some principles on which therapy can be based and, if possible, eventually to attain a rational basis in this field comparable to that in the other fields of medical treatment.

In gonorrhea in the male, one is dealing, in the majority of cases, with a local infection beginning on a mucous surface and extending into the deeper tissues and glands, but remaining confined to the organs immediately contiguous to the original site of infection. The anatomic structure of these organs bears an important relation to the course and outcome of the disease. The urethra is surrounded, from the fossa navicularis to the vesical orifice, by various glandular structures, of which the glands of Littre, the glands of Cowper, and the prostate gland are the most important. All of these glands are racemose, convoluted and of narrow lumen. The gonococcus penetrates them readily, and in a short time they become plugged with exudate or closed off by scar tissue. As a part of the researches in this laboratory, a study has been made of the anatomy of these glands in the embryo and in the adult [1] and an examination of Johnson's plates shows what a labyrinth of passages exists, and how deeply the anatomic relations must influence the clinical course of a gonorrheal urethritis.

The gonococcus calls forth a cellular exudate made up, in the early stages, mostly of polymorphonuclear leukocytes. The organism undergoes phagocytosis by these leukocytes, but it is characteristic that only a small proportion of the cells in a given exudate ingest the

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[1] Johnson, F. R., Anat. Record, in press.

gonococci, and even in these there is evidence to show that the intracellular cocci remain viable.[2] The reaction, then, by which the body seeks to overcome the gonorrheal infection, is not entirely a phagocytic one. Work with vaccines has not shown that a bactericidal immune body is effective in curing urethral infections. It is possible that the rapid development of scar tissue creates walled-off nests of infection, for example, in Littre's glands or in the prostate, where the immune forces of the body are unable to penetrate. Extensive microscopic studies of acute gonorrhea will undoubtedly throw much light on these problems. Material is now being collected in this laboratory for such study.

Gonorrhea, unlike many other infections, occurs in a locality in which it may be subjected to direct treatment with antiseptic or germicidal drugs. It is self-evident that treatment should be given at an early stage whenever possible, before the infection has embedded itself deep in the peri-urethral structures. Therapy with drugs based on the conception of their germicidal action, has had a fair measure of success. Other things being equal, drugs capable of being put in true solution and having high penetrating powers, such as those of the mercurochrome series developed in this laboratory,[3] should be more effective in reaching all the foci of infection. The first step, therefore, to be made in solving the problem of gonorrhea is to obtain reliable data concerning the germicidal value of drugs for gonococci when brought in contact with them, in order to determine what parallelism exists between germicidal activity and therapeutic effect.

PREVIOUS METHODS

A number of investigators have sought for methods of determining the efficiency of drugs against the gonococcus, and all have encountered difficulties in their work. These difficulties have resulted mainly from the uncertain and unsatisfactory growths of the gonococcus on culture mediums. This obstacle has only recently been overcome, and the reader is referred to the article by Swartz[4] in which a full discussion of recent and older culture methods will be found.

[2] Scholtz, W., Arch. f. Dermat. u. Syph., 1899, 49, p. 3; Deut. med. Wchnschr., 1905, 31, p. 935; Neisser u. Scholtz, Handb. d. path. Mikroorg., 1903, 3, p. 148.

[3] Young, H. H.; White, G. C., and Swartz, E. O.: Jour. Am. Med. Assn., 1919, 73, p. 1483.

[4] Jour. Urology, 1920, 4, p. 325.

This difficulty in obtaining large amounts and reliable growths of gonococcus has led some of those working on this problem to adopt methods based on what may be called the "antiseptic" or "inhibition" principle. Quantities of the drug to be tested are mixed with the culture medium in such a manner that known dilutions thereof are present in the successive tubes of a series varying from weak to strong. A heavy implantation of gonococci is then made in each tube, and all are cultivated in the incubator in the usual manner. The criticism of this method is that the drug acts on the organism during the entire period of culture, a condition that bears no relation to clinical use in which the drug is effective for only a limited time. Further, the presence of a constant concentration of the drug in the tube may inhibit all growth of the organisms when many or all have escaped actual killing and remain quite viable and capable of normal growth were the antiseptic substance removed. This, of course, would mean that such inhibited organisms in gonorrhea would soon multiply and reestablish the infection in the urethra; for here the drug would be removed by absorption, by dilution with exudate, by being chemically broken down, or by flushing of the urethra with urine. Therefore, it is readily seen that this method gives erroneous results, indicating germicidal powers which are too high, and that useful conclusions cannot be drawn from such tests.

The use of this method, with many of the older uncertain culture methods, adds a further element of unreliability to the results so obtained.

As is well known, the principles on which modern germicidal testing is based were enunciated by Geppert [5] and subsequently developed by Kronig and Paul,[6] Rideal and Walker,[7] Madsen and Nyman,[8] Chick and Martin [9] and others.[10] In essence, the methods of these workers consist in placing a quantity of the organisms, usually in the form of a constant or fairly constant emulsion, in a known dilution of the drug to be tested. After standing a definite length of time, a small portion, usually that adhering to a platinum loop of constant size (or 0.1 c c removed by a small pipet) is transferred to a fresh tube

[5] Deutsch. med. Wchnschr., 1891, 17, p. 797.

[6] Ztschr. f. Hyg. u. Infektionskr., 1907, 25, p. 1.

[7] Jour. Roy. Sanit. Inst., 1903, 24, p. 424.

[8] Ztschr. f. Hyg. u. Infektionskr., 1907, 57, p. 388.

[9] Jour. Hyg., 1908, 8, p. 564.

[10] Norton, J. F., and Hsu, P. H., Jour. Infect. Dis., 1916, 18, p. 180. Somerville and Walker, Pub. Health, 1905, 18, p. 385.

of culture medium which is usually plated. The small quantity of drug transferred with the organisms is supposed to be so greatly diluted in the fresh medium that it has no further action. If no growth occurs, one assumes that the organisms were killed during their contact with the solution of germicidal substance. If the gonococcus is employed in this manner, the quantity of organisms transferred to the fresh tube or medium is so small that growth is uncertain and unreliable. This is especially true with the older culture methods in which growth was uncertain under the best conditions. It has been noted that a heavy planting with gonococcus is necessary to obtain the most luxuriant growths.[4]

Steinschneider and Shäffer[11] used a mixture of two parts water and one part human serum, previously heated one-half hour to 60 C., for making their emulsions of gonococci. The emulsion was heavy, and into 2 c c of it was placed the test drug in such a manner that the desired concentration was reached. The mixture was kept at 35 C. and at the end of the desired time 3 loopfuls were removed and planted on serum agar. By this method, an appreciable quantity of drug must have been carried over to the culture medium, and the number of gonococci transferred must have been comparatively small. Few of the drugs in the series of Steinschneider and Shäffer are now in use, but in cases in which comparison can be made, their figures are not greatly different from ours. Their work is by far the best on this subject up to the present time.

Post and Nicoll[12] tested many germicides by a method in which one loopful of the organism was placed in 0.5 c c of a solution of the test drug. At the end of 1, 5, 10 and 30 minutes, and 20 hours, 1 loopful of the drug-organism mixture was transferred to blood agar and plated. The temperature of the reaction is not stated. The results show in the main low germicidal activity for the drugs tested. Argyrol, 50%, failed to kill in 30 minutes, and the gonococcus is said to grow well after suspension in distilled water for 20 hours. No mention is made of microscopic controls of the final growths. These results are at variance with the experience of ourselves and others with this organism.

Clark and Wylie[13] mixed 2 c c of a salt solution emulsion of gonococcus with 2 c c of a solution of the test drug. At the end of 5, 15 and 30 minutes, one loopful was transferred to ascitic fluid agar and plated. The temperature of the reaction was not stated. They tested a series of silver compounds and cresol and found the silver compounds comparatively ineffective. The method and checks are not described in great detail.

Fürstenau[14] used the inhibition method and also a method in which the drug was added to broth cultures of gonococcus. Transfer to the culture medium was made with a platinum loop. The temperature at which the tests were carried out is not stated. The results of this author with acriflavine show high killing power, which cannot be reconciled with any of our results.

[11] Kongress Deutsch. Dermat. Gesellschaft, Breslau, 1894, p. 156.
[12] Jour. Am. Med. Assn., 1910, 55, p. 1635.
[13] Ibid., 1911, 57, p. 394.
[14] Ztschr. f. Augenheilk., 1918, 40, p. 1.

Attempts have been made to modify the technic to make it more suitable for gonococci. Culver [15] mixed emulsions of gonococci with dilutions of the test drug and transferred a drop of the mixture to slanted mediums. He worked with only four silver compounds.

Steinschneider and Schäffer,[11] as a variant on the previously described method, took agar slants on which growths of gonococcus had been produced, and poured dilutions of the test drug directly on them. After a given time it was poured off, and a portion of the growth remaining on the agar was transferred to a new tube by means of a loop. Few figures for results by this technic are given, so that one cannot conclude how satisfactory the method was found.

DISCUSSION OF PRINCIPLES INVOLVED IN THE GERMICIDAL METHOD

Chick [16] and Chick and Martin [9] have expounded with great clearness the principles underlying germicidal action. All who have tested germicidal substances [17] find that there are great differences in the resistance of various organisms to these agents. It is therefore impossible to postulate from figures hitherto obtained with other organisms, what will be the reaction of a highly parasitic organism like the gonococcus. At the beginning of our work, we felt convinced that in order to avoid the troubles of earlier workers our method must differ from theirs in that comparatively large numbers of gonococci must be exposed to the action of the test drug, and that at the end of the test period a comparatively large proportion of these treated organisms must be transferred to the medium. In order to accomplish the latter object without carrying over too much of the test drug, the cocci would have to be freed from it in some manner. The use of the centrifuge, as first proposed by Schäffer,[18] suggested itself for this purpose.

Chick [16] has worked out the mathematical formulas for disinfection where time, concentration of test durg, or temperature is made to vary. She has also shown that the time of disinfection is a function of the number of bacteria present, following laws exactly similar to those of a chemical reaction, i. e., mass action. A definite proportion of the living organisms is killed in each unit of time. The curve is a hyperbolic one. In tests made on the gonococcus, with a view to a possible therapeutic application, the conditions should be so arranged that only the concentration of the test drug varies. All other factors should be constant or as nearly constant as possible.

15 Jour. Lab. & Clin. Med. 1918, 3, p. 487.

16 Jour. Hyg., 1908, 8, p. 92.

17 Ztschr. f. Hyg. u. Infektionskr., 1907, 25, p. 1; 57, p. 388. Jour. Roy. Sanit. Inst., 1903, 24, p. 424. Jour. Hyg., 1908, 8, p. 654. Kongress Deutsch. Dermat. Gesselschaft. Breslau 1894, p. 156. Kenwood and Hewlett, Public Health, 1906, 18, p. 462. Derby, G. S., Boston Med. & Surg. Jour., 1906, 155, p. 341.

18 Ztschr. f. Hyg. u. Infektionskr., 1894, 16, p. 189.

Temperature.—The temperature at which the drug is allowed to act on the organisms should undoubtedly be that of the body, 37.5 C.

Time.—As regards the length of time during which this contact continues: We have selected 20 minutes, since we feel that this is the longest time it may conservatively be assumed that any drug acts in gonorrheal treatment, and that in the urethra it undoubtedly acts for this length of time unless a flushing with urine occurs, even when the meatus is not held closed more than 5 minutes. It is our conviction, since time is such an important factor in germicidal action, that any drug intended to kill gonococci in the urethra should be allowed to act for the longest possible time.

Number of Organisms in the Emulsion.—The criteria for constancy in the number of organisms used were similar to those of Chick.[16] Cultures of equal age were used grown on medium always prepared in the same manner and emulsified in the same way with the same quantity of 0.85% sterile salt solution. The age of the strain is of minor importance, for our results with old laboratory strains and with freshly isolated strains were the same. It would undoubtedly be more accurate to count the gonococci in the emulsion. This, however, is difficult and time consuming, owing to the tendency of the organisms to cohere, and since our tests checked well on different days, we did not consider it essential to do so. As stated, the emulsions to be used contained a large number of gonococci. This necessarily means that complete disinfection in a given time will require a higher concentration of the test drug than if a small number of organisms were used in the emulsion. But, as indicated above, this fact, if kept well in mind, does not affect the usefulness of the test in any way.

Number of Organisms Transferred.—The use of the centrifuge enables one to wash the organisms free of the test drug at the end of the stated period, and at the same time, by concentrating them at the bottom of the centrifuge tube, makes it possible to transfer to the culture medium almost all of the organisms originally introduced into the test solutions, instead of say $\frac{1}{3000}$ as with a platinum loop.[19] Chick[16] has shown that with certain germicides, especially those containing the heavy metals, the quantity carried over on a platinum loop to the culture medium may, even when very small, exert an important inhibitory effect if the number of organisms surviving the action of the test drug is small.

[19] Shohl, A. T., and Janney, J. H., Jour. Urology, 1917, 1, p. 211.

Advantages of the Centrifuge Method.—This method is calculated to overcome the objection mentioned above in two ways: First, the organisms are washed free of the test drug as thoroughly as desired, and second, almost all of the organisms in the original emulsion may be transferred to the culture tube. The implantation will still be liberal enough to overcome any small inhibitory effect, even if a considerable proportion of organisms have been killed. For example, let us assume that 10,000,000 organisms are introduced in a test solution which is of such a strength that 99% are killed in 20 minutes. If a loopful, containing $\frac{1}{3000}$ of the mixture be taken, only about 33 living organisms will be transferred. If, on the other hand, by the centrifuge method one transfers 80% of the organisms originally introduced, 80,000 survivors will be planted on the culture tube instead of 33.

Method of Culture After Action of Drug.—After the treated gonococci are transferred to the culture medium, there still remains to be considered the interpretation of the resulting growths. In testing ordinary organisms, it is customary to transfer to melted agar, which is immediately poured into Petri dishes. The number of colonies developing can then be counted in those cases in which some of the organisms have survived. In the case of the gonococcus, there are several objections to this procedure. Since the growth of gonococcus occurs only on the surface of solid mediums,[4] a plate would not indicate truly the number of surviving organisms, but only those which did not come to rest in the depths of the medium. The necessity for using mediums containing uncoagulated human protein would make the pouring of plates, especially in lengthy series of tests, difficult and time consuming. The great susceptibility of the gonococcus to heat, making it necessary to keep agar for the plates nearly at its solidification point, would increase the difficulty. Lastly, any germicidal action falling short of completion would be useless in the urethra. For these reasons, it seems simpler and more expedient to inoculate the treated organisms on slanted solid mediums and to consider as giving positive results only those tubes in which no growth occurs.

The Presence of Protein.—The presence of some protein material in the drug-organism mixtures is desirable, since in practical use the drugs must always act in the presence of protein. This may be attained by the addition of sterile hydrocele or ascitic fluid or blood serum. If the emulsion is made directly from the protein-containing medium, it will contain about 0.4% hydrocele or ascitic fluid.

Comparison with Other Organisms.—It will readily be seen that a method evolved from these principles will be favorable to the organism rather than to the drug. If, however, it is a method by which end-points for different drugs can be readily obtained and by which reliable and consistent growths are to be had, this is not an objection to its practical use. In order to compare the susceptibility of the gonococcus with that of other organisms, however, one would have to perform other tests using the same technic and using the other organisms in place of the gonococcus, taking care to keep the number of organisms in the emulsion comparable to the number used in the gonococcus emulsions. In case the end-points were too high with this technic, necessitating the use of undesirably concentrated solutions of the test drug, the time of the reaction could be extended as desired, calculating back to a period of twenty minutes by the formula of Chick.[16]

Preliminary Studies.—All of the considerations treated in the last section did not make themselves felt at once in the beginning of our work. Having secured, through the work of Swartz,[4] an eminently satisfactory method of growing gonococci with great profusion and certainty, we turned first to the antiseptic or inhibitory method. Little work was done along this line, as the technic was considered faulty from a theoretical point of view. The results thereof have been rejected. One of us (S) thereupon devised a method, later found to be similar to that utilized by Steinschneider and Shäffer,[11] in which the solution to be tested was poured on a 24-hour slant culture of gonococci. After remaining a given time, it was poured off, the culture washed with 0.85% salt solution, and transfers made to fresh medium. This method also has theoretic objections since the thickness of the layer of gonococci is an unknown quantity. The amount transferred varies, since more or less of the layer of gonococci may be removed by the test fluid or the subsequent washings. The results were inconstant, and the need of a more exact and better controlled method was felt.

It was determined to utilize the gonococci in the form of an emulsion. The emulsion may be prepared from slants or from fluid cultures. We have utilized slants throughout our work because growth of the gonococcus occurs in both fluid and solid mediums on the surface,[4] and a greater number can be procured from the larger surface of the slant. The remaining steps of the technic were developed according to the principles outlined in the preceding section.

DESCRIPTION OF METHOD

Our method is as follows:

1. All the tubes and pipets and all the solutions to be used, except those of the test drug, are sterilized. The emulsion is prepared by introducing 7½ c c 0.85% sterile NaCl solution into a 24-hour culture of gonococcus on slanted ascitic or hydrocele fluid agar.[4] The growth is scraped off into the salt solution, using a platinum needle. The emulsion is poured into a tube containing glass beads, and fitted with a 1 c c pipet running through a cotton stopper. After shaking, it is placed in a water bath at 37.5 C. If more than 7.5 c c are needed, a second or third tube may be prepared in the same way and added to the emulsion. Since gonococci do not survive indefinitely in salt solution, the emulsion is always discarded at the end of one hour and a fresh one substituted. While the dilution is made with physiologic salt solution, a small amount of protein will be taken up from the surface of the medium and the "water of condensation." This is allowed to remain.

2. Beginning with a strong solution of the test drug, which is presumably sterile, dilutions thereof are made with sterile pipets and tubes, using sterile distilled water as a diluent. These dilutions are made of twice the strength in which it is desired to test the drug. One c c of each dilution is placed in a sterile, cotton stoppered, properly labeled, 15 c c centrifuge tube. These centrifuge tubes are warmed to 37.5 C. before inoculation.

3. Into each tube is put 1 c c of the gonococcus emulsion. This makes the total contents of the tube 2 c c and reduces the test drug to the desired concentration. The tube is then placed in the water bath at 37.5 C. and allowed to stand 20 minutes. It is our custom to inoculate the tubes at 5-minute intervals. This allows time, at the end of the test period for each tube, to centrifuge, wash, and transfer.

4. At the end of 18 minutes, the tube is removed from the water bath, placed in a warm metal holder, and centrifuged rapidly for 2 minutes in a high-power centrifuge. At the end of 2 minutes the gonococci will be found to have settled to the bottom of the tube much more completely than is the case with, for example, B. coli or Staphylococus aureus, and the supernatant fluid will be quite clear. This is poured off, replaced by 2 c c of warm 0.85% NaCl solution, the gonococcus thoroughly mixed with the salt solution, and the whole

centrifuged a second time. We usually wash once, but with the more concentrated test solutions, we wash two or three times. One may wash as often as desired.

5. After the final centrifugalization, the last wash fluid is poured off, the gonococci remaining as a compact mass in the tip of the tube. With a properly shaped loop, they can be removed almost completely, since they show a tendency to cohere. Some drugs destroy this tendency to a certain extent, but in any case, a liberal planting can be procured. The organisms so obtained are spread over the surface of a fresh slant of ascitic or hydrocele fluid agar, which is then heated, corked and incubated according to the technic described by Swartz.[4]

6. The tubes are read at the end of 24 hours, 48 hours, and 4 days. Growth usually appears in 24 hours, but with some drugs it may be delayed in some tubes one or two days. All growths are counted as positive, regardless of the number of colonies present. The growth in all tubes is examined in a smear by means of a Gram stain, to detect the possible presence of contamination.

7. At the end of 7 days, all tubes showing no growth are reinoculated from a fresh, viable culture of gonococcus. Each tube should show a growth after this planting, proving that not enough of the test drug was carried over with the transfer loop to make the culture medium unsuitable for growth.

We have utilized this method in testing a large series of drugs. We have found that duplicate tests on different days check within reasonable limits of experimental error. The end-point is sharp and definite. Suitable controls included in each series have always shown profuse growth, proving that the manipulations involved in the method have no deleterious influence on the gonococcus. The method is satisfactory as regards contamination. In spite of the fact that the solutions of the test drug are not sterilized, we have had not more than 7 contaminated series, or about 3.5% of the entire number. All the final growths have been carefully examined microscopically. We have not tested any drugs which precipitate protein, since the results of such tests would be of little value, and we can see no reason for the use of such drugs for their germicidal value.

DISCUSSION

The results of tests made by this method will give definite figures bearing on the ability of various drugs to kill the gonococcus when

brought in contact with it. Such figures will show that certain drugs used in the treatment of gonorrhea do not destroy gonococci. Their beneficial effects are exercised through other means. The figures will also show which of the drugs used or proposed for therapeutic purposes is the most efficient germicide for gonococcus. Trials will determine the exact relation between these laboratory tests and clinical effectiveness.

It is obvious that the effective strength of any drug in the urethra will depend not only on the concentration in which it will kill gonococci, but also on the strength in which it may be used in the urethra without irritation. This relation may be expressed as a factor obtained by dividing the concentration in which a given drug will kill gonococci in a given time by the concentration in which it may be used in the urethra. Thus, if a drug will kill in a concentration of 1 : 20,000 and may be used in a concentration of 1 : 100, the factor will be 200, while another drug which kills at 1 : 40,000, but will be irritating in any strength over 1 : 1,000 has a factor of only 40.

While we cannot at the present time predict the clinical efficiency of a given drug from laboratory tests, it is quite possible that at a future date we will be able to do so. The development of a reliable method such as is described in this paper is a necessary and important step forward toward this goal.

At present, we are in a position to state that the relative efficiency of the drugs commonly used in the treatment of gonorrhea as germicides, is quite different from that commonly supposed, and also that certain organic mercurial drugs tested in this laboratory, which are colorless, water soluble, penetrating, comparatively nontoxic, and do not coagulate protein, are more efficient germicides for the gonococcus than any others tested.

The results of our tests using the method herein described are being published in a series of papers.

SUMMARY

The application of the principles of germicidal action to tests using the gonococcus as the test organism has been discussed.

A new, practical and reliable method for making germicidal tests with the gonococcus as the test organism has been presented.

THE THERMAL DEATH POINT IN RELATION TO TIME OF TYPICAL THERMOPHILIC ORGANISMS

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By thermal death point in relation to time is meant the length of time at different temperatures necessary to completely destroy a definite concentration of spores in a medium of known hydrogen-ion concentration.

Several exact methods are in use for determining the death point of various organisms subjected to the influence of moist heat and early investigators have determined with considerable precision the resistance to heat of some of the common micro-organisms. The usual procedure for the determination of the thermal death point of vegetative forms is to expose a suspension in broth or salt solution to the action of a given temperature for 10 minutes. In the case of spores the thermal death point is generally understood as the time necessary to effect the death of all the spores when exposed to 100 C.

Little accurate work has been published on the effect of high temperatures (above 100 C.) on resistant spores, and no reliable method for such determination can be found in the bacteriologic literature. Whenever it has been desired to determine the time above 100 C., varying amounts of calcium chloride have been added to the water in the bath, or the autoclave has been used.

Lawrence and Ford [1] and Laubach, Rice and Ford,[2] in establishing the thermal death points of the spores of some resistant aerobes, subjected suspensions in broth culture to various temperatures both in the Arnold sterilizer and in an autoclave. During their work neither the effect of the time necessary to attain the desired temperature nor of the time allowed for the cooling of the cultures was considered.

The importance, especially in the sterilization of canned foods, of knowing the conditions affecting, and the time necessary for, the complete destruction of resistant spores at high temperatures has led to the introduction of a new method which gives reliable and accurate results for the determination of the thermal death point of heat resis-

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[1] Jour. Bacteriol., 1916, 1, p. 273.

[2] Ibid., p. 493.

tant spores. We give a description of this method together with some results obtained which show not only the effect of different temperatures on the time necessary to destroy a definite suspension of spores of certain typical thermophilic bacteria, but also the influence of different concentrations of the same spores in medium of the same and varying hydrogen-ion concentration.

TECHNIC

Apparatus.—We used the following apparatus: De Khotinsky electric bath with a thermo-regulator attachment; turbine stirrer; motor $\frac{1}{70}$ H. P.; liquid of high boiling point; special culture tube—soft glass,[3] closed at one end, 7 mm. (inside diam.) by 250 mm. long with a 1 mm. thickness of wall; Wassermann test-tube rack; wire holders for suspending culture tubes in bath.

The mediums used in this work consist of nutrient agar and broth, prepared according to standard methods, and the juices of the following canned foods: corn, peas, spinach, string beans, pumpkin, sweet potatoes, and beets. These juices are prepared by expressing the contents of the canned food through a flannel cloth by means of a fruit and lard press. The juice is autoclaved at 15 pounds for 5 minutes and the supernatant fluid decanted, tubed and sterilized in the autoclave. This increases the hydrogen-ion concentration of the juice to some extent. The P_H value of some of the juices employed was therefore somewhat lower than is usually found with the corresponding canned foods.

Nutrient agar slants are inoculated with a pure culture of the organism to be tested and grown at the optimum temperature of that specific organism until a luxuriant growth occurs along the inoculated area (24-48 hours). In the case of rapidly growing organisms a shorter period in the incubator will suffice. Usually the growth from two agar slant tubes will produce an adequate number of organisms per cubic centimeter but when dealing with bacteria that grow much less abundantly, as is the case with some thermophiles, 4 to 8 tubes should be used.

[3] Dr. E. C. Dickson has called the attention of the writers to the fact that the heating in soft glass tubes of unbuffered acid and aqueous solutions, lowers their hydrogen-ion concentration, which does not occur when pyrex glass tubes are used.

The writers find that the various food juices used in the work described in this article remain quite constant in P_H value when heated in soft glass tubes. The buffer substances, presumably, take care of the alkali dissolved from the glass and in soft glass tubes the hydrogen-ion concentration of the food juices does not increase during the heating period as it does in pyrex glass tubes. Better duplicates of the P_H value are secured with pyrex glass than with soft glass tubes.

When the cultures are ready, a smear of each growth is stained and examined microscopically in order to ascertain that pure cultures of the right micro-organisms are obtained. Carefully observing aseptic precaution throughout, a portion of a test tube of sterile nutrient broth, P_H 7 is poured over the surface of the first slant culture, shaking the fluid in such a way as to bring the micro-organisms into suspension. If the culture is not easily washed from the medium, a sterile platinum loop or pipet may be used to remove the growth, care being taken not to cut into the medium and mix the fragments with the bacterial suspension. The suspension thus obtained is poured on the surface of the second culture, bringing this into suspension and repeating the process until the entire series of cultures has been washed, using fresh portions of sterile broth when the suspension has become sufficiently concentrated.

The final bacterial suspension is transferred to a sterile thick-walled flask and incubated for one week at the same temperature as the slants. At the end of the incubation period the inoculated flasks are removed from the incubator and placed in the icebox. After 48 hours they are heated to 85 C. for 15 minutes to kill all vegetative forms, cooled immediately and placed again in the icebox to prevent the germination of spores. This is the stock suspension from which portions are used for the determination of the thermal death point of spores. Nine c c of the medium to be used are inoculated with 1 c c of the stock suspension. This dilution gives satisfactory results and does not appreciably alter the hydrogen-ion concentration of a medium between ranges of P_H 4.5 and 7.0. The special culture tubes described under apparatus, which have been previously wrapped with heavy brown paper in packages of 10 each and sterilized, are inoculated with 1 c c of this suspension of spores. These tubes are of the smallest diameter that can be conveniently used in order to reduce to a minimum the time necessary for the heat to penetrate the center of the medium. They stand a sudden change of temperature over a wide range and can be subjected to the shock of sudden cooling without injury. It has been found that in less than 20 seconds the temperature of the tubes is reduced to that of the ice bath, even when the tubes have been heated at 140 C. These inoculated tubes are then sealed off to within 2 inches of the surface of the liquid, placed in a Wassermann rack and held in a bath of ice water until ready to be heated.

The thermal death point of the spores at 100 C. is determined by immersing the sealed tubes in an open water bath vigorously boiling

at 100 C. The De Khotinsky electric bath is used in maintaining a constant temperature, using paraffin oil or a hydrogenated oil for temperatures of 105 to 140 C. The adjustment to the desired temperature is effected by means of a thermoregulator attachment and the temperature kept uniform throughout the bath by means of a turbine stirrer operated by a $\frac{1}{70}$ H. P. motor. A thermometer graduated to 0.1 C., standardized by the Bureau of Standards, is kept at a constant level in the oil to record the temperature. In our experiments the temperature of the oil bath did not vary more than 0.1 + or — after calculating for the stem correction at all temperatures. For temperatures of 105 C. to 125 C., inclusive, the sealed tubes are immersed in a bath of boiling water for 15 seconds before being subjected to the oil bath adjusted at the desired temperature. When a temperature above 125 C. is used, the cultures are exposed in an intermediary oil bath of 115 to 120 C. for 15 seconds. When it is desired to determine the time necessary to destroy a definite number of spores at temperatures at or below boiling, no preliminary heating is given.

Before immersing the sealed tubes in this adjusted bath, the temperature is increased one half of one degree in order to compensate for the loss in the temperature due to the immersion of the tubes in the oil. During the 15 seconds which are allowed for the heat to reach the center of the tubes, before recording the time, the temperature of the oil bath due to the cooling effect of the tubes drops to the temperature at which it was previously adjusted and thereafter remains constant.

A series of tubes, each containing the same concentration of spores, is exposed to the desired temperature for definite periods of time. At the end of each period a tube is removed and immediately placed in a bath of ice water in order to prevent further action of the heat on the spores of the bacteria. When cold, these tubes are placed in an icebox and held until the sterility of the medium can be determined.

Sterility is determined by inoculating nutrient broth with the contents of the heated tubes and incubating for one week. Growth is confirmed by making streak plates from the nutrient broth cultures. In most cases, when spores have survived the heating, growth occurs within 48 hours, and in no case has growth occurred after the fourth day. Some workers have found that the germination of spores which have been subjected to high temperatures is delayed for unusually

long periods of incubation. Burke [4] reported that spores of B. botulinus in brain medium survived heating in the autoclave at 15 pounds' pressure for 10 minutes, the growth in subcultures being determined only after 53 days' incubation. Experiments in connection with this work carried out according to the method outlined above gives evidence of no retarded germination of spores of typical thermophilic organisms heated at high temperatures, and tubes showing no growth after one week's incubation are considered sterile.

The initial concentration of the spores is determined according to standard methods, the plates being incubated for 48 hours. The hydrogen-ion concentration of the medium is determined colorimetrically. Electrometric measurements made to check the colorimetric method showed close agreement in every case. The determination of the entire series is made on the same day so as to obtain constant and comparable condition.

EXPERIMENTAL

The method described was used to determine the relationship between time and temperature necessary for the complete destruction of the spores of some typical thermophilic bacteria. The temperatures were 100 to 140 C. at intervals of 5 C., and the medium was the juice pressed from canned corn, P_H 6.1. The same concentration of spores which had been subjected to the same preliminary treatment was used at each temperature, and all the conditions standardized as far as possible. The organisms were true thermophiles isolated from cases of spoilage in a variety of canned foods. The presence of these organisms in the canned product without exception was due to their great heat resistance and their ability to withstand the usual sterilizing processes.

Although these obligate thermophiles were found to be the most heat-resistant of all the organisms studied, their power of heat resistance varied greatly for the different species, as can be readily seen from table 1. The range in resistance of those tested varied from 3 to 23 minutes at 120 C. Spores of the same organism varied in resistance to heat under special conditions and could be increased greatly. For example, one type was increased from 12 to 23 minutes at 120 C. within 6 months through repeated artificial cultivation. The same increase in resistance was accomplished by cultivating the surviving spores after understerilizing a suspension. By careful selection, a very resistant type was produced. The retention of stock sus-

[4] Jour. Am. Med. Assn., 1919, 72, p. 88.

pensions for 6 months in the icebox did not alter appreciably the resistance of the spores.

The results of several experiments over a lapse of several months under different conditions shows that the time necessary to destroy completely a definite concentration of spores is greatly reduced as the temperature is increased.

Table 1 gives the time in minutes necessary to destroy a known concentration of spores of some typical thermophilic bacteria at temperatures from 100 to 140 C., giving in the left hand column the

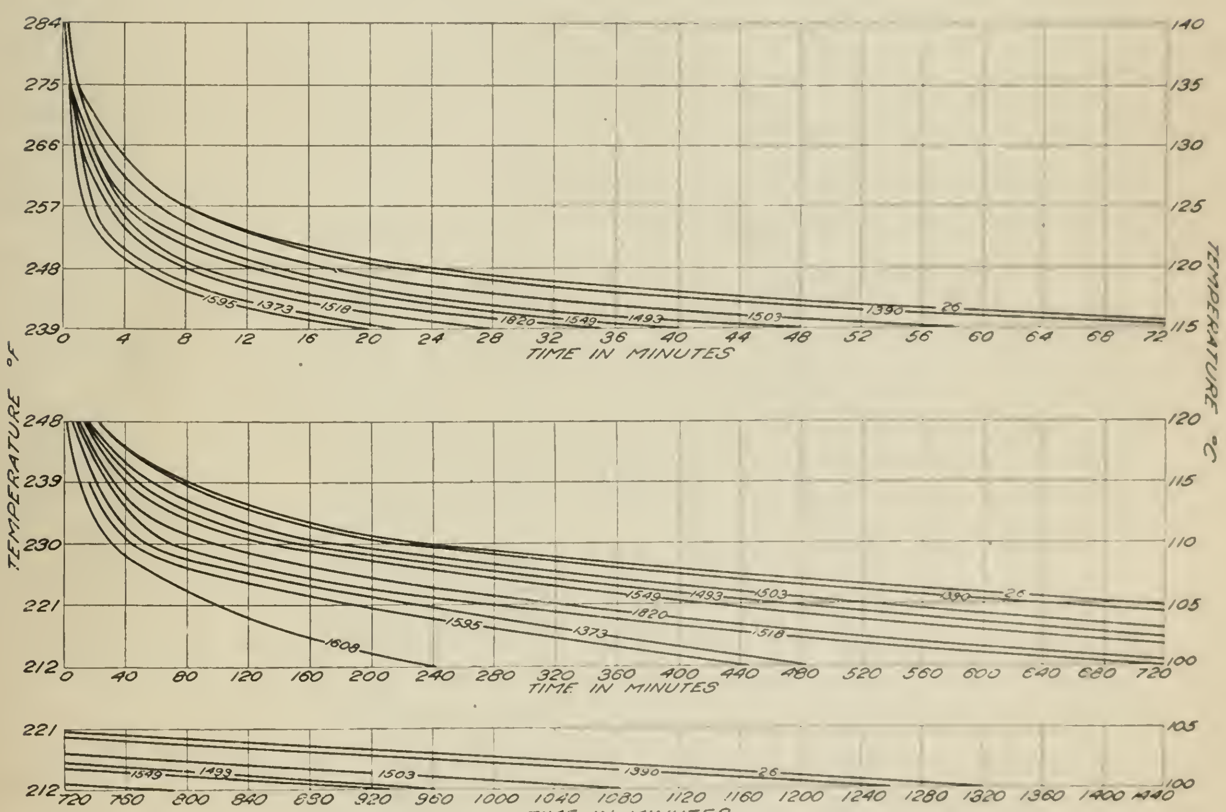


Fig. 1.—Thermal death point curves of typical thermophilic organisms, Ph 6.

longest time during which growth occurred and in the right hand column the first time growth did not occur, showing the destruction of all the spores present.

Figure 1 shows the effect of the temperature on the sterilizing time as represented by curves. Each curve is designated by the laboratory number of the organism with which it was obtained. It will be noted that the curves are approximately parallel so that a knowledge of the time necessary to destroy 50,000 spores of an organism of this class at a given temperature enables us to construct the entire curve.

TABLE 1

Time Necessary to Destroy the Spores of Typical Thermophiles at Temperatures Stated

Culture Number	Number of Bacterial Spores per C c	Corn Juice P_H 6.1	Minutes Required to Destroy Spores in Corn Juice P_H 6.1 at																	
			100 C.		105 C.		110 C.		115 C.		120 C.		125 C.		130 C.		135 C.		140 C.	
			+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—
1608	12,000	6.1	210	240	95	100	30	32	4	5	2	3	...	1						*
1356	85,000	6.1	300	360	130	150	32	35	12	15	3	4	1	1.25	...	0.75				
1595	100,000	6.1	420	450	220	225	45	48	18	20	4	5	1	1.5	0.5	0.75	....	0.5		*
1373	6,500	6.1	450	480	225	235	50	53	22	24	5	6	1.5	2	1	1.25	0.5	0.66	....	0.5 *
1518	25,000	6.1	660	720	250	270	60	70	25	30	7	8	2	3	1	1.25	0.5	0.66		*
1820	25,000	6.1	720	750	300	360	90	95	30	35	8	9	3	3.5	1	1.5	0.5	0.66		*
1476	70,000	6.1	750	780	380	400	110	115	33	36	9	10	3.5	4	1.5	1.75	0.5	0.66	....	0.5
1492	225,000	6.1	780	840	360	390	100	105	35	38	10	11	3.5	4	1.5	1.75	0.5	0.66		
1549	50,000	6.1	900	930	400	420	130	135	35	40	12	13	3.5	4	1.5	2.0	0.66	0.75		*
1493	4,500	6.1	930	960	450	480	145	150	45	50	13	14	4	5	1.5	2.0	0.75	1.0	....	0.5 *
1503	100,000	6.1	1020	1080	500	525	170	175	55	58	16	17	5	6	2.0	2.5	0.75	1.0	....	0.5 *
4109	12,000	6.1	1080	1140	550	575	180	185	70	72	17	18	6	7	2.5	3.0	0.75	1.0	0.5	0.75
1592	115,000	6.1	1140	1200	575	600	185	190	66	70	18	19	6	7	2.5	3.0	0.75	1.0	0.5	0.75
1390	35,000	6.1	1200	1260	630	660	210	215	75	78	21	22	7	8	3.0	3.5	1.0	1.25	0.75	1.0 *
26	200,000	6.1	1260	1320	660	690	220	225	80	84	22	23	7	8	3.0	3.5	1.0	1.25	0.75	1.0 *

* Organisms used for Chart 1.

The thermal death point of the spores of resistant organisms is influenced by such factors as hydrogen-ion concentration, initial concentration of spores, age and the condition of spore and heat penetration. We shall consider the first two factors.

INFLUENCE OF HYDROGEN-ION CONCENTRATION

The influence of the hydrogen-ion concentration on the thermal death point of spores is shown in table 2 and figure 1. This was obtained by heating a number of spores at a constant temperature in food juices of different P_H values. The work thus far has been done solely on food juices, owing to the necessity of obtaining immediate results to assist the commercial canner in the processing of canned foods. This phase of the problem is to be continued by varying the hydrogen-ion concentration in the same medium. The results so far indicate, however, that the hydrogen-ion concentration of the medium inoculated with a suspension of spores is most important, and that the time necessary for the destruction of bacterial spores is largely dependent on this factor. It is believed that the time necessary to destroy a definite number of spores in string bean juice or corn juice will be nearly the same as that in any solution with the same P_H value, provided the other influencing factors remain constant. Table 2 shows the time necessary to destroy a specified number of spores in the following juices: corn, peas, sweet potatoes, pumpkin, spinach, string beans and beets, when heated at 120, 115, 110 and 100 C. The suspensions for the same organisms were treated similarly at the four temperatures, using the same initial concentration of spores in each case. The results are given in the same way as in table 1, showing the longest time at which growth occurred and the first time at which no growth occurred. The hydrogen-ion concentration of each juice employed as determined electrometrically, is as follows: corn 6.1, peas 5.3, sweet potato 5.0, spinach 5.0, string beans 5.0, beets 4.7, and pumpkin 4.5.

Figures 2, 3, and 4 give curves showing the relation of time to hydrogen-ion concentration of representative thermophilic organisms. In Figures 3 and 4 it will be noted that three distinct results were obtained at P_H 5. This is explained by the fact that three of the juices employed (sweet potato, spinach and string beans) had a P_H value of 5. It is obvious from this that hydrogen-ion concentration is not the only important factor that has a bearing on thermal death point. Notwithstanding this, the curves show a marked relationship between the hydrogen-ion concentration and thermal death point.

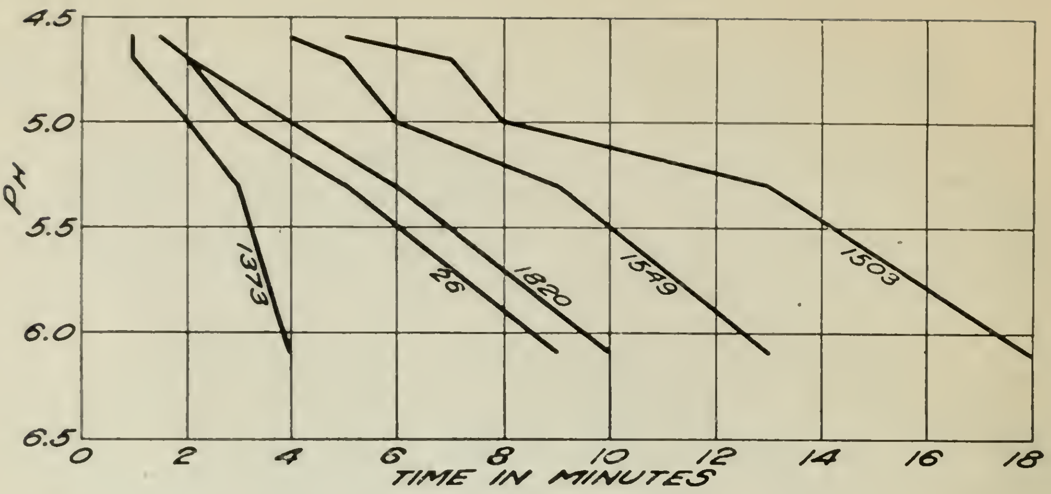


Fig. 2.—Influence of hydrogen-ion concentration of various food juices on the time necessary to destroy spores heated at 120 C.

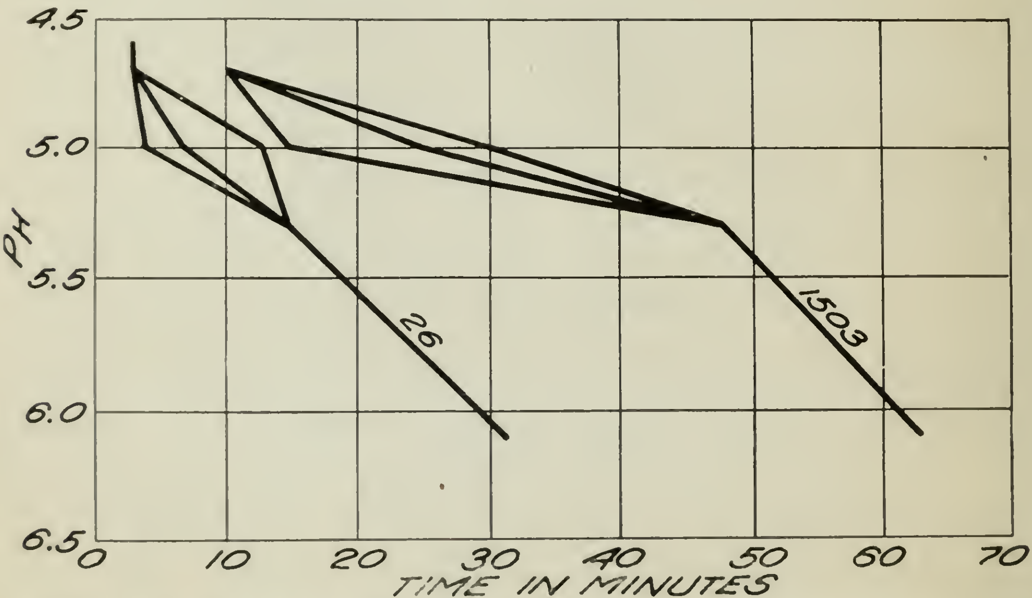


Fig. 3.—Influence of hydrogen-ion concentration of various food juices on the time necessary to destroy spores heated at 115 C.

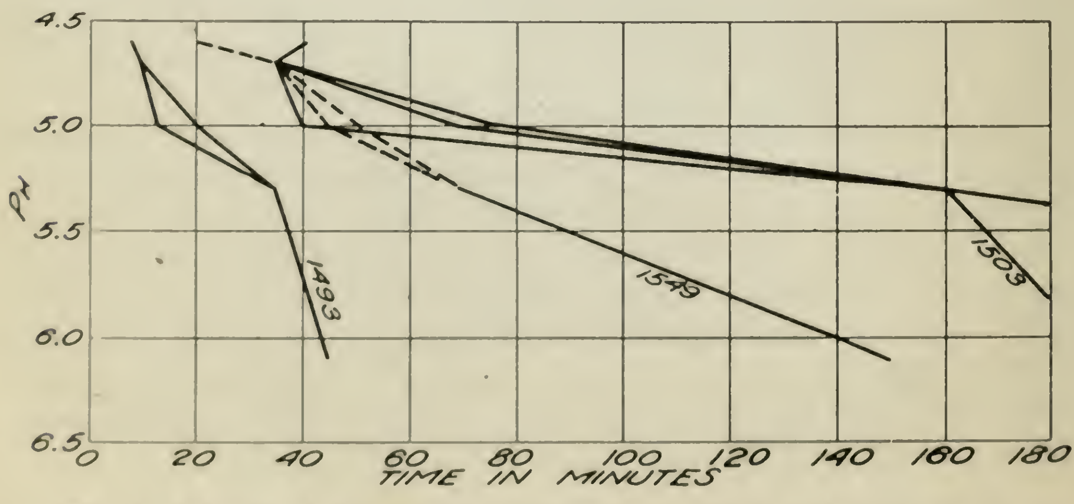


Fig. 4.—Influence of hydrogen-ion concentration of various food juices on the time necessary to destroy spores heated at 110 C.

TABLE 2

THERMAL DEATH POINT OF THE SPORES OF TYPICAL THERMOPHILES IN MEDIUMS OF DIFFERENT HYDROGEN-ION CONCENTRATION WHEN HEATED AT TEMPERATURES STATED

	Culture Number	Number of Spores per C c	Minutes Required to Detroy Spores Heated in													
			Corn Juice P_H 6.1		Pea Juice P_H 5.3		Sweet Potato Juice P_H 5.0		Spinach Juice P_H 5.0		String Bean Juice P_H 5.0		Beet Juice P_H 4.7		Pumpkin Juice P_H 4.5	
			+	—	+	—	+	—	+	—	+	—	+	—	+	—
120 C.	1503	150,000	17	18	12	13	7	8	6	7	7	8	6	7	4	5
	4109	40,000	16	17	9	10	9	10	4	5	6	7	4	5	4	5
	1390	60,000	13	14	9	10	7	8	3	4	3	4	4	5	3	4
	1549	50,000	12	13	8	9	5	6	3	4	3	4	4	5	2	3
	1492	10,000	11	12	4	5	2	3	2	3	2	3	2	3	2	3
	1476	100,000	9	10	4	5	2	3	1	2	2	3	1	2	2	3
	1820	100,000	9	10	5	6	3	4	3	4	3	4	1	2	1	2
	26	100,000	8	9	4	5	2	3	2	3	2	3	1	2	1	2
	1518	10,000	7	8	6	7	2	3	4	5	4	5	2	3	4	5
	1356	85,000	5	6	4	5	2	3	2	3	2	3	1	2	1	2
	1508	500,000	5	6	3	4	1	2	1	2	1	2	1	2	1	2
	1493	150,000	4	5	3	4	1	2	1	2	1	2	0.5	1	1	2
	1373	100,000	3	4	2	3	1	2	1	2	1	2	0.5	1	0.5	1
	1608	25,000	2	3	2	3	1	1.5	1	1.5	1	1.5	0.3	0.5	1	1.5
115 C.	1503	150,000	60	63	45	48	28	30	13	15	20	25	9	10	10	10.5
	4109	40,000	58	60	40	45	25	28	9	10	15	18	9	12	10	11
	1390	60,000	55	58	35	40	25	28	6	7	6	7	10	12	6	7
	1549	50,000	35	40	25	30	15	18	10	12	10	12	12	15	5	6
	1492	10,000	30	35	12	15	9	10	6	7	6	7	4	5	4	5
	1476	100,000	33	35	8	10	5	6	2	3	5	6	2	3	5	6
	1820	100,000	30	32	15	18	10	11	9	10	9	10	3	4	9	10
	26	100,000	28	31	12	15	11	13	3	4	5	7	2	3	2	3
	1518	10,000	20	25	20	25	12	13	10	12	10	12	12	13	10	12
	1356	85,000	12	15	10	12	6	8	3	4	3	4	3	4	3	4
	1508	500,000	12	15	10	12	4	5	2	3	2	3	2	3	2	3
	1493	150,000	18	20	10	12	6	7	3	4	3	4	2	3	2	3
	1373	100,000	12	15	7	8	4	5	3	4	3	4	2	3	1	2
	1608	25,000	4	5	4	5	2	2.5	1.5	2.0	1.5	2.0	1	1.5	1.5	2
110 C.	1503	150,000	180	190	150	160	70	75	35	40	65	70	30	35	35	40
	4109	40,000	160	165	90	100	70	75	28	30	28	30	25	28	28	30
	1390	60,000	130	140	90	95	65	70	20	25	20	25	25	28	20	25
	1549	50,000	140	145	60	70	45	50	40	45	40	45	30	35	18	20
	1493	10,000	110	120	40	45	20	25	20	25	20	25	12	15	12	15
	1476	100,000	110	120	35	40	12	15	4	5	12	14	4	5	12	14
	1820	100,000	110	120	70	75	30	35	25	30	25	28	15	18	25	28
	26	100,000	110	120	60	65	26	30	10	12	12	14	10	12	10	12
	1518	10.000	60	70	60	65	25	30	30	35	30	35	25	30	25	27
	1356	85 000	45	50	40	45	20	25	15	20	15	20	15	20	10	12
	1508	500,000	35	40	30	35	4	5	4	5	4	5	4	5	4	5
	1493	150,000	40	45	30	35	15	20	11	13	11	13	8	10	7	8
	1373	100.000	30	35	20	25	5	6	4	5	4	5	2	3	4	5
	1608	25,000	30	32	25	30	3	4	2	3	2	3	2	3	2	3
100 C.	1503	150,000	1140	1200	960	1020	300	360	180	210	300	360	180	210	180	210
	4109	40,000	1140	1200	600	720	300	360	150	180	150	180	180	210	150	180
	1390	60,000	900	960	800	840	...	...	240	270	240	270	210	240	240	270
	1549	50,000	900	960	720	780	...	...	120	180	180	240	180	210	60	90
	1492	10,000	840	900	600	720	...	...	180	210	180	210	120	150	120	150
	1476	100,000	720	840	540	600	...	...	30	60	120	150	30	40	120	150
	1820	100,000	1200	1320	720	840	...	...	180	240	180	240	150	180	180	210
	26	100,000	840	900	540	600	270	300	180	210	150	180	120	150	120	150
	1518	10,000	720	740	690	720	120	150	150	180	150	180	120	150	120	150
	1356	85,000	420	480	360	420	120	150	120	150	120	150	120	150	90	120
	1508	500,000	300	360	180	240	...	...	60	75	75	90	30	45	60	75
	1493	150,000	480	540	480	510	135	150	120	135	120	135	120	135	45	60
	1373	100,000	300	360	300	360	60	90	45	60	45	60	45	60	45	60
	1608	25,000	180	240	150	180	30	45	25	30	20	25	15	20	25	30

TABLE 3

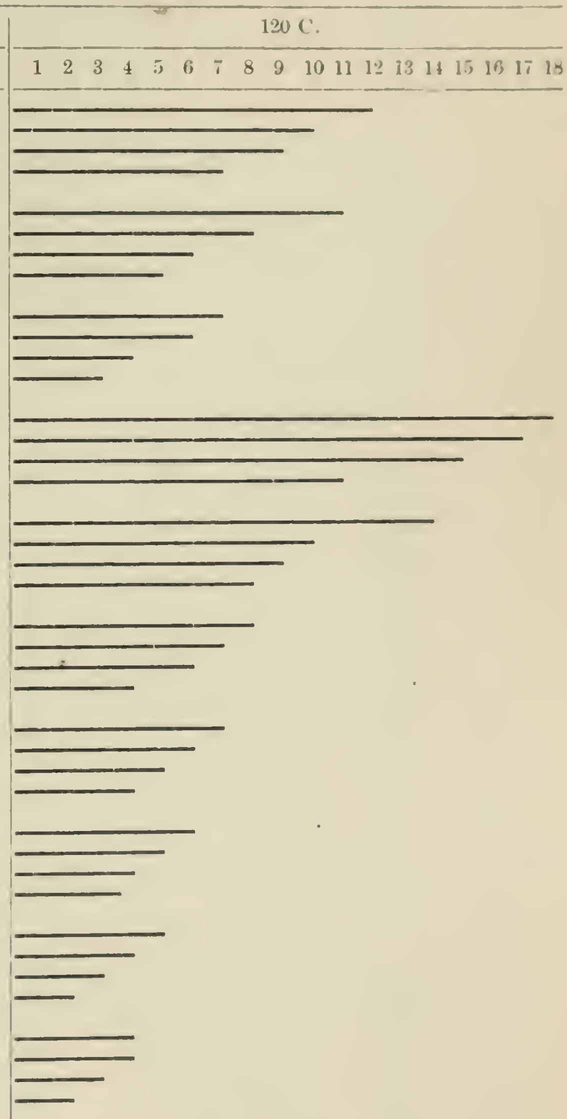
EFFECT OF INITIAL CONCENTRATION ON TIME NECESSARY TO DESTROY THE SPORES OF TYPICAL THERMOPHILES AT TEMPERATURES STATED

Culture Number	Number of Bacterial Spores per C c	P_H Value	Minutes Required to Destroy Spores of Different Concentrations																	
			100 C.		105 C.		110 C.		115 C.		120 C.		125 C.		130 C.		135 C.		140 C.	
			+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—
26	200,000	6.0	1260	1320	660	690	220	225	80	84	22	23	7	8	3.0	3.5	1.0	1.25	0.75	1.0
26	20,000	6.0	1080	1140	550	575	180	185	70	72	17	18	6	7	2.5	3.0	0.75	1.0	0.50	0.75
26	2,000	6.0	1020	1080	520	540	170	175	55	60	16	17	5	6	2.0	2.5	0.66	1.0	....	0.66
1592	115,000	6.0	1140	1200	575	600	185	190	66	70	18	19	6	7	2.5	3.0	0.75	1.0	0.5	0.75
1592	10,000	6.0	960	1020	480	495	155	160	48	50	15	16	4	5	2.0	2.5	0.75	1.0	....	0.66
1493	175,000	6.0	1020	1080	500	525	170	175	55	58	16	17	5	6	2.0	2.5	0.75	1.0		
1493	5,000	6.0	900	960	450	480	145	150	45	50	13	14	4	5	1.5	2.0	0.75	1.0	....	0.5

TABLE 4

EFFECT OF INITIAL CONCENTRATION ON TIME NECESSARY TO DESTROY THE SPORES OF TYPICAL THERMOPHILES IN CORN JUICE AT 120 C.

Culture Number	Initial Concentration of Spores	P_H Value	Minutes Required to Destroy Spores of Different Initial Concentrations, 120 C. +	120 C. —
26	40,000	6.0	11	12
	3,800	6.0	9	10
	440	6.0	8	9
	130	6.0	6	7
1421	33,000	6.0	10	11
	3,500	6.0	7	8
	450	6.0	5	6
	80	6.0	4	5
1390	26,000	6.0	6	7
	3,000	6.0	5	6
	400	6.0	3	4
	74	6.0	2	3
4109	130,000	6.0	17	18
	13,000	6.0	16	17
	1,300	6.0	14	15
	130	6.0	10	11
4112	50,000	6.0	13	14
	5,000	6.0	9	10
	500	6.0	8	9
	50	6.0	7	8
1476	120,000	6.0	7	8
	12,000	6.0	6	7
	1,200	6.0	5	6
	120	6.0	3	4
1820	140,000	6.0	6	7
	14,000	6.0	5	6
	1,400	6.0	4	5
	140	6.0	3	4
1356	130,000	6.0	5	6
	13,000	6.0	4	5
	1,300	6.0	3½	4
	130	6.0	3	3½
1493	200,000	6.0	4	5
	20,000	6.0	3	4
	2,000	6.0	2	3
	200	6.0	1	2
1508	350,000	6.0	3	4
	35,000	6.0	3	4
	3,500	6.0	2	3
	350	6.0	1	2



INFLUENCE OF CONCENTRATION OF SPORES

Schmidt,[5] working with spores of the black-leg bacillus, observed that the larger the number of spores the longer the period of heating necessary to destroy them, concluding that the resistance of the spores increases with the number. Gage and Stoughton,[6] in a study of the heat resistance of B. coli, found that the majority of bacteria in a

[5] Ztschr. f. physik. Chemie., 1906, 21, p. 414.

[6] Science, 1906, 23, p. 216.

TABLE 5

Effect of Initial Concentration on Time Necessary to Destroy the Spores of Typical Thermophiles in Corn Juice at Temperatures Stated

Culture Number	Initial Concentration of Spores	P_H Value	Minutes Required to Destroy Spores of Different Initial Concentrations					
			120 C.		125 C.		130 C.	
			+	—	+	—	+	—
26	26,500	6.3	18	19	5	6	2.5	3
	2,650	6.3	15	16	3.5	4	2.5	3
1503	40,000	6.3	18	19	5	6	3	3.5
	4,000	6.3	15	16	4	5	2.5	3
1820	130,000	6.3	16	17	5	6	3	3.5
	13,000	6.3	15	16	4	5	2.5	3
1390	85,000	6.3	12	13	4	5	2	2.5
	8,500	6.3	9	10	3	4	2	2.5
1373	4,300	6.3	16	17	5	6	2.5	3
	430	6.3	14	15	4	5	2	2.5
	43	6.3	12	13	4	5	1.5	2
1592	80,000	6.3	14	15	4	5	2	2.5
	8,000	6.3	12	13	4	5	1.5	2
1518	35,000	6.3	8	9	2	3	0.5	1
	3,500	6.3	7	8	1.5	2	1	1.5
1476	15,000	6.3	10	11	3	4	2	2.5
	1,500	6.3	9	10	2	3	1.5	2
	16	6.3	6	7	2	3	1	1.25
1574	40,000	6.3	13	14	4	5	2	3
	4 000	6.3	10	11	4	5	2	2.5
	350	6.3	9	10	3	4	2	2.5
4109	90,000	6.3	16	17	5	6	2.5	3
	9,000	6.3	14	15	4	5	2	2.5
	750	6.3	11	12	3.5	4	2	2.5
	130	6.3	7	8	2	3	1	1.5
1595	380,000	6.3	5	6	3	4	1.5	2
	38,000	6.3	4	5	1.5	2	...	1
	3,500	6.3	3	4	1	1.5	...	0.75
	350	6.3	2	3	...	1		
1321	35,000	6.3	12	13	3	4	1.5	2
	3.500	6.3	10	11	2	3	1	2
	560	6.3	8	9	2	3	1	2
1356	22.500	6.3	16	17	5	6	2.5	3
	2,250	6.3	14	15	4	5	2	3
	456	6.3	12	13	3.5	4	1.5	2
1508	780,000	6.3	3	4	1	1.5	...	0.5
	78,000	6.3	2	3	1	1.5		
1492	50,000	6.3	10	11	3.5	4	1.5	2
	5,000	6.3	8	9	2	3	1.5	2
	700	6.3	7	8	2	3	1	1.5
1493	50 000	6.3	16	17	5	6	2	2.5
	5,000	6.3	11	12	3	4	2	2.5
1549	15,000	6.3	7	8	1.5	2	1	1.5
	1,500	6.3	6	7	2	2.5		
	400	6.3	5	6	1.5	2	0.5	0.75

culture are destroyed by a certain temperature in a relatively short time, while a few individuals will survive much higher temperatures. Eijkman,[7] noticing the variations in rate of death when using different amounts of bacteria, concluded that this was due to substances that were given off into the medium by the killed bacteria.

TABLE 6

Effect of Initial Concentration on Time Necessary to Destroy the Spores of Typical Thermophiles in Corn Juice at 125 C. and 118 C.

Culture Number	Initial Concentration of Spores	P_H Value	Minutes Required to Destroy Spores of Different Initial Concentrations			
			125 C.		118 C.	
			+	−	+	−
26	65,000	6.3	5.5	6		
	22,000	6.3	5	5.5		
	2,260	6.3	5	4.5		
	290	6.3	2	2.5		
1421	27,500	6.3	3	4		
	2,760	6.3	2.5	3		
	500	6.3	2	2.5		
	123	6.3	1.5	2		
1390	43,000	6.3	2	3		
	16,000	6.3	2	2.5		
	2,850	6.3	1.5	2		
	318	6.3	1.5	2		
26	7,000	6.3	...	...	26	29
	1,100	6.3	...	...	21	24
	110	6.3	...	...	16	19
	12	6.3	...	...	5	8
1390	55,000	6.3	...	...	16	19
	14,000	6.3	...	...	16	18
	1,250	6.3	...	...	12	14
	175	6.3	...	...	8	10

The effect of the initial numbers of spores of certain thermophiles on the time required for their complete destruction is determined by diluting the stock culture several times in mediums of the same hydrogen-ion concentration. A series of 9 c c tubes of sterile medium are prepared and dilutions made as follows: 1 : 10, 1 : 100, 1 : 1,000, 1 : 10,000, etc., until the final dilution contains less than 10 spores per c c. The thermal death point is determined in the usual manner, and without exception the result shows that the larger the number of spores present in the medium, the longer the time necessary to destroy them. Table 2 gives the results as obtained with different initial concentrations in corn juice, P_H 6, at temperatures from 100 to 120 C. Similar suspensions were used at each temperature and the results

[7] Biochem. Ztschr., 1908, 11, p. 12.

are comparable in every respect, showing again the relation of time to temperature.

The remaining tables show the influence of the concentration of spores on the time necessary to destroy the organisms at various temperatures. The results given in the several tables are not comparable with each other as they were obtained with organisms which had been

TABLE 7

Effect of Initial Concentration on Time Necessary to Destroy the Spores of Typical Thermophiles in Corn Juice at 115 C., 110 C., 105 C.

Culture Number	Initial Concentration of Spores	P_H Value	Minutes Required to Destroy Spores of Different Initial Concentrations					
			115 C.		110 C.		105 C.	
			+	−	+	−	+	−
26	45,000	6.3	62	65				
	4,300	6.3	30	35				
	400	6.3	25	28				
	40	6.3	20	22				
1390	35,000	6.3	40	42				
	2,550	6.3	24	26				
	275	6.3	19	21				
	58	6.3	8	10				
1421	35,000	6.3	46	50				
	1,000	6.3	26	28				
	100	6.3	15	18				
	13	6.3	8	10				
26	3,700	6.3	34	35				
	610	6.3	30	33				
	61	6.3	23	25				
	3	6.3	15	16				
1390	70,000	6.3	40	42				
	4,500	6.3	28	30				
	600	6.3	22	23				
	65	6.3	11	13				
1421	12,000	6.3	36	39				
	1,500	6.3	27	30				
	123	6.3	18	20				
	18	6.3	10	13				
26	90,000	6.3	...	...	200	210		
	18,500	6.3	...	...	200	205		
	3,200	6.3	.....	...	190	200		
	250	6.3	...	...	170	180		
1390	125,000	6.3	...	...	190	195		
	31,000	6.3	...	...	160	170		
	2,250	6.3	...	...	160	165		
	175	6.3	...	...	150	160		
26	170,000	6.3	...	...	...	...	600	660
	90,000	6.3	...	...	...	...	600	630
	11,500	6.3	..	...	...	...	540	570
	9,000	6.3	...	...	...	...	540	560
	1,100	6.3	...	...	...	...	510	540
	85	6.3	...	...	...	...	480	510
1390	120,000	6.3	...	...	...	...	510	540
	100,000	6.3	...	...	...	...	500	520
	12,000	6.3	...	...	...	...	450	480
	1,100	6.3	...	...	...	...	440	460
	130	6.3	...	...	...	...	400	420
	20	6.3	...	...	...	...	360	390

cultured for various lengths of time and hence were of different resistance.

CONCLUSIONS

A new method for determining the thermal death point of resistant spore-bearing bacteria at high temperature, which gives accurate results, is described.

The time necessary to destroy a known suspension of spores in medium of known hydrogen-ion concentration decreases as the temperature increases. For example, 200,000 spores per c c of culture 26 are destroyed in 1,320 minutes in boiling water (100 C.), 690 minutes at 105 C., 225 minutes at 110 C., 84 minutes at 115 C., 23 minutes at 120 C., 8 minutes at 125 C., 3.5 minutes at 130 C., 1.5 minutes at 135 C., and 1 minute at 140 C. Nineteen thermophilic organisms studied in this connection show the same relationship between time and temperature.

TABLE 8

TIME NECESSARY TO DESTROY SPORES OF TYPICAL THERMOPHILES AT 120 C.

Culture Number	P_H	No. of Bacterial Spores per C c	Minutes Required to Destroy Spores in Corn Juice at 120 C.																													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	28	30	32	36
1608	6.0	12,000	+	⊕	−	−	−	−	..	−	..	−																				
1356	6.0	85,000	..	+	+	−	−	−	−	−	..	−	..	−																		
1595	6.0	100,000	..	+	+	+	−	−	−	−	..	−	..	−																		
1373	6.0	6,500	..	+	..	+	+	−	−	−	..	−	..	−																		
1518	6.0	25,000	..	+	..	+	+	+	⊕	−	−	−	..	−	..	−																
1820	6.0	25,000	..	+	..	+	..	+	+	+	−	−	−	−	..	−																
1476	6.0	70,000	..	..	..	+	..	+	+	+	⊕	−	−	−	..	−																
1492	6.0	225,000	..	..	..	+	..	+	..	+	+	⊕	−	−	−	..	−															
1549	6.0	50,000	..	..	..	..	..	+	..	+	..	+	+	+	−	−	−	−	..	−												
1493	6.0	4,500	..	..	..	..	..	+	..	+	..	+	+	+	+	−	−	−	..	−	..	−										
1503	6.0	100,000	..	..	..	..	..	..	..	+	..	+	..	+	+	..	+	+	−	−	..	−	..	−	..	−						
4109	6.0	12,000	..	..	..	..	..	..	..	..	..	+	..	+	..	+	..	+	+	−	−	−	..	−	..	..	−	..	−			
1592	6.0	115,000	..	..	..	..	..	..	..	..	..	..	..	+	..	..	+	..	⊕	+	−	−	−	−	..	−	..	−	..	−		
1390	6.0	35,000	..	..	..	..	..	..	..	..	..	..	..	+	..	..	+	..	..	+	..	+	+	−	−	−	−	−	..	−	..	−
26	6.0	200,000	..	..	..	..	..	..	..	..	..	..	..	..	..	+	..	+	..	+	..	+	+	+	−	−	..	−	..	−	..	−

+ = growth in 15 hours' incubation at 55 C.
⊕ = growth in 48 hours' incubation at 55 C.
− = no growth in 7 days' incubation at 55 C.

The hydrogen-ion concentration influences the time necessary to destroy a known suspension of spores at a given temperature. As the P_H value is increased, the time required for complete destruction is decreased.

The initial concentration of spores per c c affects the time necessary to sterilize a medium of known hydrogen-ion concentration at a given temperature. The larger the number of spores present in a medium, the longer the time necessary to destroy them.

THE EPIDEMIOLOGY OF HEMOLYTIC STREPTOCOCCUS CARRIERS, BASED ON A STUDY OF SUBGROUPS *

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The hemolytic streptococci came into great prominence during the World War, as they were one of the most common causes of death in the respiratory diseases. In the investigation of the possible modes of spread of the streptococcus as complicating these infections numerous studies have been made on the incidence of carriers. However, most of the carriers thus diagnosed have been identified as harboring organisms belonging to the hemolytic streptococcus group as a whole rather than to any of the subgroups. Advance in bacteriology and epidemiology rests on the subdivision of a group of organisms, as in the case of the pneumococci. In this paper an attempt is made to approach the problem of epidemiology through such a study of subgroups. Subdivisions of hemolytic streptococci based on their fermentation reactions, is possible and is of value, since striking and suggestive results were obtained by grouping carriers and by similarly grouping organisms obtained from the air of hospital wards—in other words, from the environment.

The serologic subgrouping of streptococci has given suggestive results in the hands of Havens,[1] and of Dochez and his co-workers,[2] but on account of technical considerations it was not felt advisable to attempt this method of classification for the purposes of this paper.

All the strains studied in this paper were isolated at Columbus Barracks, Ohio, during February and March, 1920. The fermentation reactions were done at the Army Medical School. Columbus Barracks was selected as the place of making the survey, since it is a recruit depot and afforded opportunity for the comparison of the incidence of carriers among men arriving from civil life with that prevailing among the permanent personnel at the post. It was also possible to reculture recruits after a brief period in the service in

[1] Jour. Infect. Dis., 1919, 25, p. 315.

[2] Dochez, Avery and Lancefield, Jour. Exper. Med., 1919, 30, p. 179.

order to ascertain whether conditions prevailing at an army post during peace time caused any increase in the carrier incidence. The general data thus obtained was felt to be of enough interest to warrant its inclusion in the paper. The Barracks had at the time of this work a soldier population of approximately 1,300. An average of 40 to 50 recruits were being enlisted daily.

TECHNIC

The technic [3] made official for army use during the war was followed. Sterile swabs were rubbed with moderate pressure over the tonsils or fossae. These were spread over the surface of blood-agar plates within 20 minutes and incubated. Beef infusion agar was used, P_H 7.6, to which 10% of its volume of defibrinated human blood was added. Twelve to fifteen c c of medium were used per plate. On the following morning hemolytic colonies were transferred to beef infusion broth, which after 24 hours' growth were tested for hemolytic activity by mixing 0.5 c c of the broth culture with an equal volume of a 5% suspension of washed blood cells and placing in water bath at 37 C. for 2 hours.

The hemolytic character of the organisms was later confirmed, using pour plates made of rabbit blood. With the exception of a few strains which were lost or not saved for further study, all the strains considered in this work were shown to produce the beta type of hemolysis, as described by Brown.[4]

The question arises as to the value of positive and negative results as detected by the technic described. Davis [5] believes that practically everybody harbors the hemolytic streptococcus. If this is correct, a negative result would have much less significance than otherwise. However, Nichols and Bryan,[6] using the same method as Davis, that is, the culturing of tonsils that had been removed, found hemolytic streptococci in only 75% of cases. Ruediger,[7] in 1906, stated that while the hemolytic streptococcus was frequently found in the throat, it could not be regarded as a normal inhabitant. This statement, I believe,

[3] Methods for the Isolation and Identification of Streptococcus Hemolyticus, U. S. Army, 1918.

[4] Monographs of the Rockefeller Institute for Medical Research, 1919, No. 9.

[5] Jour. Am. Med. Assoc., 1920, 74, p. 317.

[6] Ibid., 1918, 71, p. 1813.

[7] Jour. Infect. Dis. 1906, 3 p 755.

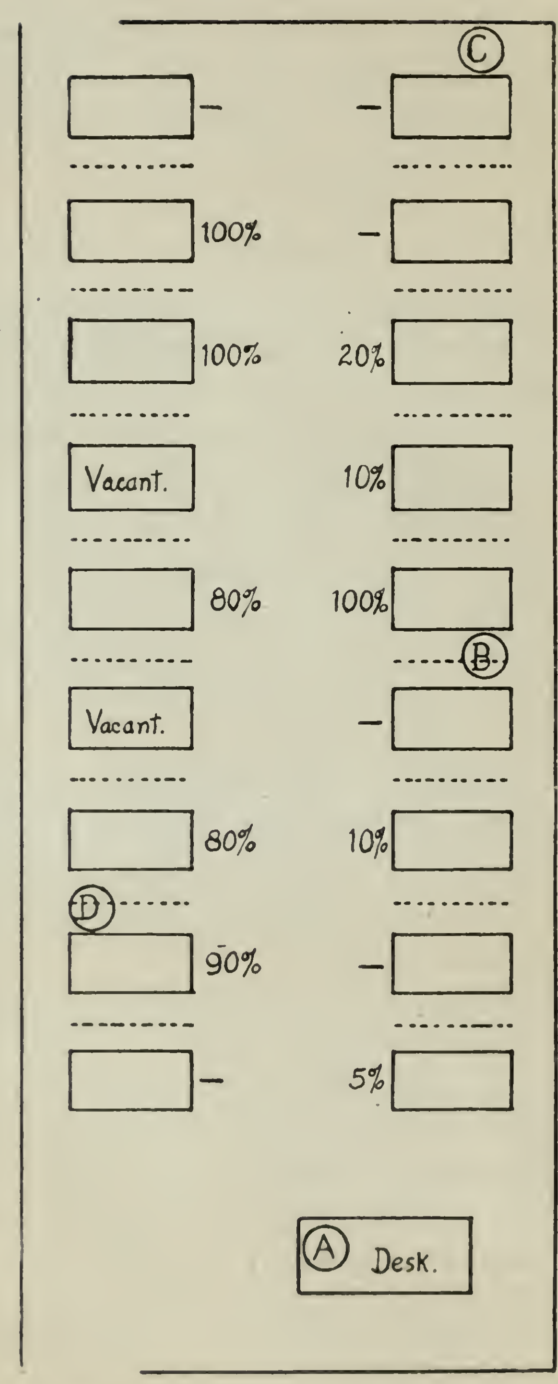

Diagram of ward 1, showing the relationship of positions in which blood agar plates were exposed to patients. The dotted lines represent screens, which were used to cubicle the ward. Approximate Scale: 1 inch = 12 feet.

still represents our present knowledge of the subject. Although it is true that some lightly infected cases may fail to be detected, this fact does not invalidate the method as a whole.

A word of caution should be said in regard to a possible error if one attempts to identify the hemolytic streptococcus simply from the appearance of a throat culture on a blood-agar plate. We have frequently encountered a hemolytic bacillus, the colonies of which on rabbit blood closely resemble those of typical hemolytic streptococci. This organism seems to be strictly hemoglobinophilic, and possibly belongs to the B. influenzae group, several hemolytic strains of which have been described by Rivers.[8] The organism is considerably larger, however, than the B. influenzae usually encountered.

The classification of Holman[9] was followed in the fermentation reactions. He classified the strains encountered by us thus:

	Lactose	Mannit	Salicin
St. infrequens	+	+	+
St. pyogenes	+	—	+
St. anginosus	+	—	—

All strains were also tested against inulin. Only one inulin fermenter was encountered in the total of 203 strains tested. This organism grew in long chains, was bile insoluble, and produced no capsules. Otherwise it fermented as St. pyogenes.

The medium used in the fermentation tests was horse serum 1 part, distilled water 4 parts, to which was added 1% of Andrade indicator and 1% of the test sugar. After the sugar was dissolved, the medium was tubed and autoclaved for 10 minutes at 10 pounds' pressure. Streptococci grew well in this medium, even before the addition of the test substances.

Before being tested, all strains were plated and single colonies transferred to broth. The fermentation tubes were inoculated with 0.1 c c of the broth cultures and incubated 24 hours. A preliminary reading was then made, and smears made from each tube on blood-agar plates. Tubes and plates were then returned to the incubator. No results were considered unless the blood-agar plate showed the profuse growth of a streptococcus in pure culture. In case of lack of growth or of contamination the test was repeated. Final readings were made on the seventh day.

[8] Bull. Johns Hopkins Hosp., 1920, 31, p. 50.
[9] Jour. Med. Res., 1916, 34, p. 377.

The organisms from the air were isolated by the exposure of blood-agar plates, for known periods of time. These plates were placed on desks or bedside tables. More improved methods of air examination have, of course, been described, but the results furnished by this method are believed to be valuable, besides having the advantage of great simplicity.

RESULTS OF CULTURES

Throat cultures were made on recruits as they arrived at the barracks and as they left. The length of stay at the barracks varied from 5 to 13 days. Cultures were similarly made from the throats of a group of soldiers from the permanent personnel of the post. The latter group all had had one or more years' service. The results are shown in table 1.

TABLE 1

INCIDENCE OF STREPTOCOCCUS CARRIERS AMONG RECRUITS AND AMONG PERMANENT PERSONNEL

	Number Cultured	Number Positive	Proportion, Percentage
Recruits arriving	470	79	17
Recruits leaving	175	28	16
Permanent personnel	99	20	20

It is seen from this table that the incidence of carriers among recruits having spent a short period at the post shows no increase over the rate prevailing among them on arrival. These rates are slightly less than that among the permanent personnel of the post, but the difference is not believed to be of significance. The incidence of 17% shown by the recruits arriving for enlistment can perhaps be taken as an index of the civilian rate. On this basis it is seen that the carrier ratio at the post was approximately the same as that among civilians.

It is interesting to compare these figures with the work of Levy and Alexander,[10] who found that recruits arriving at Camp Zachary Taylor (near Louisville, Ky.), in 1918, showed a carrier incidence of only 14.8%, while a group of men who had been at the camp 6 months or more showed an incidence of 83.2%. The figures obtained at Columbus show, however, that such an increase is not an invariable rule. The greater proportion of the recruits examined at Columbus (79%) were from urban communities.

[10] Jour. Am. Med. Assoc., 1918, 70, p. 1827.

The incidence of carriers among those with abnormal throats was higher than among the rest of the group; 34% of those showing hypertrophied tonsils or injection of the pharynx were positive. The influence of pathologic conditions of the throat were especially marked in relation to the persistence of positive cultures. Of the 175 men cultured twice, 163 were considered as having normal throats. Of these seven, or 4%, were positive on both occasions. The remaining 12 were noted as having hypertrophied tonsils or injection, or both. Five of them, or 38%, were positive on the 2 cultures. Ten cases had had previous tonsillectomies, all of whom were negative for hemolytic streptococci.

TABLE 2

Classification of the Hemolytic Streptococci According to Their Fermentation Reactions

Source	Number of Strains	Pyogenes		Infrequens		Anginosus	
		Number	Proportion, Percentage	Number	Proportion, Percentage	Number	Proportion, Percentage
Recruits arriving	65	53	82	10	15	2	3
Recruits leaving	27	23	85	4	15	0	
Permanent personnel	20	13	65	1	5	6	30
Hospital detachment	7	6	86	0	0	1	14
Measles	14	6	43	8	57	0	
Other patients	32	30	94	2	6	0	
Air	34	30	88	4	12	0	
Total	199	161	80	29	15	9	5

The rest of the organisms isolated from throats were obtained from a survey of 37 men on duty at the hospital, of whom 9 (24%) were positive, and from 81 patients in the hospital, of whom 48% were positive. Among the latter the highest incidence was in 17 cases of measles, of whom 14, or 82%, were positive, and 3 cases of scarlet fever, all of whom were positive. The lowest percentage of carriers was in a ward of 18 A. E. F. patients with facial wounds. These showed an incidence of 11%.

The classification of these organisms and those obtained from the air is shown in table 2.

Compared with the recruits, the permanent personnel and the hospital detachment show a high percentage of anginosus strains. Thirty per cent of the carriers among the permanent personnel were infected with anginosus and none of the recruits leaving had this strain. This

makes it probable that any new carriers produced among the recruits during their stay at the Barracks were infected from their companions rather than from the men permanently on duty at the post.

The anginosus subgroup is also conspicuous by not occurring among the strains isolated from patients. At Columbus, at least, it could be practically disregarded as a cause of disease.

The high percentage of St. infrequens occurring in measles patients is striking. It is possible that this resulted simply from new patients with measles becoming infected with the strains of infrequens present in the ward, rather than to special predilection of these organisms for patients with measles.

The infrequens strains from two other patients (table 2) came, one from a case of tonsillitis and the other from bronchopneumonia following measles. Since the remaining strains from patients were all pyogenes, it is not necessary to give in detail the conditions in which they occurred.

The following instances are cited since they tend to show that an organism causing an infection or a carrier state in an individual remains true to type: As mentioned, 12 recruits were positive on both the occasions that they were cultured. The subgroups of the strains isolated from each coincided exactly on the two cultures, 10 of them being pyogenes and 2 infrequens.

Two of the measles cases, which were complicated with otitis media, furnished information of the same nature. The strains from the throat and from the ears were in both instances infrequens. Another patient in the surgical ward had an infected appendectomy wound, from which St. pyogenes was isolated, as well as from his throat.

The question of the persistence of a subgroup of the organism in an individual is of great importance in relation to the epidemiology of streptococcus infections and has a direct bearing on all efforts to trace the source of an infection. From the instance cited, for example, we are justified in concluding that the patients in the measles wards infected with St. pyogenes had no relation to the measles patients who developed otitis media, since the latter were caused by St. infrequens. Also, as far as the evidence goes, it is possible that the infection of the surgical wound was caused by the strain in the patient's own throat. This possibility of course could not be considered had the strains from the throat and from the wound belonged to different subgroups.

ORGANISMS RECOVERED FROM THE AIR

The presence of a large number of patients in the hospital with positive throat cultures offered opportunity to investigate the occurrence of hemolytic streptococci in the air. A cultural survey was made on the patients at the same time the plates were exposed. Other colonies developing at 37 C. in 24 hours were counted, as well as the hemolytic streptococci.

Table 3 shows the places in which plates were exposed and the number of colonies developing. As a matter of interest, the calculation for the number of hemolytic streptococci settling per hour per square meter is included in the table.

TABLE 3

STREPTOCOCCI ON BLOOD-AGAR PLATES EXPOSED IN VARIOUS PLACES

Place	Number of Patients	Number of Carriers	Number of Plates Exposed	Time Each Plate Was Exposed, Hours	Total Number Colonies	Total Number of Colonies of Hemolytic St.	Hemolytic St. per Sq. Meter per Hour
Ward 1, medical.........	16	10	16	4	5,735	59	144
Ward 11, venereal........	6	1	2	2	550	6	236
Ward 2, surgical.........	18	5	3	2	420	5	131
Ward 6a, surgical........	4	1	2	2	160	2	79
Ward 7, surgical.........	20	1	2	2	375	1	39
Ward 7, surgical*........	19	0	8	4	2,835	0	

* Exposure 3 days after carrier at first present in ward had been discharged.

In an experiment by O. Teague, similar to the above, reported by Nichols,[11] the rate of settling out in a ward containing carriers was 8 hemolytic streptococci per square meter per hour. However, his plates were exposed at night after all the patients had retired. The plates mentioned in table 3 were exposed in the afternoon between 1 and 5 o'clock, during which time there was considerable moving about due to the ward routine, and especially to the serving of dinner and supper. This may account in part for the much higher figures obtained at Columbus.

The organisms isolated had the same cultural and morphologic characteristics as organisms isolated from carriers or patients. They belonged to the same subgroups as the organisms from patients. Furthermore, they occurred in greatest number in wards in which there were carriers, and were absent or relatively few in number in

[11] Jour. Lab. & Clin. Med., 1920 5, p. 502.

a ward in which there were no carriers.[7] These considerations make it entirely reasonable to assume that the organisms from the air were identical with the organisms occurring in the patients.[8]

From table 2 it will be noted that 4 of the organisms obtained from the air were infrequens. These were all from the same ward (ward 11) and included all the organisms recovered from this ward that were tested against the sugars. Only one carrier was detected in this ward, and his strain was St. pyogenes. Unfortunately, cultures were not made from the throats of the ward personnel. Hence, it is believed that in all probability there was a carrier of St. infrequens among the personnel. However, even in the absence of having discovered the origin of these strains, their presence all in one ward is believed to emphasize the practical importance, from the standpoint of epidemiology, of grouping streptococci according to their fermentation reactions.

TABLE 4

Hemolytic Streptococci from the Air in Ward 1

Position	March 20		March 21		March 22		March 23		Total Hemolytic St.	Total Other Colonies
	Hemolytic St.	Other Colonies	Hemolytic St.	Other Colonies	Hemolytic St.	Other Colonies	Hemolytic St.	Other Colonies		
A	4	210	5	400	5	550	2	430	16	1,590
B	4	220	2	450	4	450	0	250	10	1 3 0
C	7	200	4	550	0	325	2	250	13	1,325
D	5	250	8	500	2	250	5	550	20	1,550

All the other organisms isolated from the air were pyogenes.

It is advisable to consider ward 1 in some detail. The illustration contains a diagram of the ward. Each bed was cubicled by means of a sheet on a movable frame, which extended about 3 feet above the level of the bed. A minus sign next to a bed means that the patient occupying it had a negative throat culture. The figures in percentage next to the other beds, except those unoccupied, indicate the proportion of the total number of colonies on the throat cultures that were hemolytic streptococci. The positions in which blood-agar plates were exposed are indicated by capital letters enclosed in circles.

Plates were exposed in each position daily for 4 hours over a period of 4 days. A case of scarlet fever had developed in this ward a few days previously, hence the ward was under quarantine and no changes occurred in the patients. The results are shown in table 4.

An analysis of this table shows that hemolytic streptococci were generally distributed throughout the air of the wards, and that cubicles did not prevent their free circulation. Position "C" is the farthest removed from any of the carriers, yet the number of colonies of hemolytic streptococci recovered here is practically the same as in the other positions.

The presence of the organisms in the air may have been due to bacteria or droplets containing bacteria emitted by the patients, or these droplets—saliva—and mucus may have settled to the floor, dried, and later passed into the atmosphere as dust. Possibly the latter factor played the major part, since this would perhaps more satisfactorily explain the general distribution. The presence of hemolytic streptococci in the dust of floors has been noted by several observers. Alexander [12] found them in sweepings from the floors of barracks or hospital wards, as did also Cummings and Spruit.[13] The latter observers, however, were unable to demonstrate them at a level more than 2 inches above the level of the floor. It is perhaps reasonable to assume, if hemolytic streptococci exist on the floor, or especially 2 inches from the floor, that they will, under certain conditions, be carried into the general atmosphere as dust.

The ease with which streptococci spread from the throat of one patient to others has been noted by several observers, especially Cole and MacCallum [14] and Levy and Alexander.[10] The latter studied the problem carefully and found that, in order to keep the throats of measles patients who entered the hospital with negative cultures from becoming positive, it was necessary to put them in separate wards. When patients with negative cultures were put in wards with patients having positive throat cultures many of them became infected, despite the most rigid precautions, including cubicling, masking when not in cubicle, careful watching, discipline, etc. It is possible that the infection of these patients occurred by means of the air at long range, as distinguished from droplet infection at short distance. In my opinion the ease with which streptococcus infection occurs cannot be explained only on the basis of direct contact of individuals, or indirectly by drinking and eating utensils, or by droplet infection at short range, however important these factors may be.

[12] Jour. Am. Med. Assoc., 1918, 70, p. 775.
[13] Mil. Surg., 1920, 46, p. 391.
[14] Jour. Am. Med. Assoc., 1918, 70, p. 1146.

SUMMARY AND CONCLUSIONS

The incidence of carriers at an army post during peace times was not greater than that in civil life. New recruits spending a short period of time at this army post did not show an increase in the carrier ratio.

Pathologic conditions of the pharynx, such as hypertrophied tonsils, tend to prolong the state of a hemolytic streptococcus carrier.

The classification of streptococci, according to their reactions on the sugars, is believed to be of great importance from the standpoint of epidemiology, and should be done in every case in which a more thorough knowledge of the streptococci isolated from patients, carriers or the enviroment is desired.

It is believed that streptococcus infections cannot be explained on the basis of contact alone. Streptococci were found to be distributed generally in the air of a ward containing patients with positive throat cultures. The presence of these organisms in the air probably explains the ease with which streptococcus infections take place. Their number is also probably influenced by the amount of dust in the room. This reemphasizes the necessity of measures to allay dust and of keeping patients with negative throat cultures in separate wards.

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